

***In vitro* studies on drug- induced metabolic alterations.**

By

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ABSTRACT

Over the last few decades some mechanisms of diseases, especially those which are associated with recently described dysregulation of the adipose tissue due to certain medications/drugs, have been discovered, shedding light on the pathophysiology of obesity-associated metabolic alterations. Investigation of the functions of distinct cytokines, overall redox status, transcriptional regulation and protein expression of crucial adipogenic markers may provide new comprehension of the pathophysiology of obesity, diabetes and cardiovascular diseases, as well as specific targets for future therapeutic approaches. This study was particularly designed with the knowledge that certain medications and drugs such as paracetamol, caffeine, rilpivirine, estradiol and β -naphthoflavone (BNF) may contribute significantly to the process of adipogenesis, broadly termed drug-induced metabolic alterations. Furthermore, the present study investigated the combined effects of some of these drugs for comparison.

The results from the treatment of adipose cells with caffeine, paracetamol and β -naphthoflavone either alone or in combination have shown anti-adipogenic effects of caffeine, paracetamol and β -naphthoflavone through inhibition of adipogenesis and enhancement of the lipolytic process. A combination of β -naphthoflavone with caffeine and paracetamol was further shown to attenuate triglyceride accumulation.

This study also investigated the inflammatory status of 3T3-L1 human pre-adipocytes when treated with different concentrations of either rilpivirine or estradiol alone and in combination with β -naphthoflavone (BNF). The results show that either rilpivirine or estradiol individually or during their combination can evoke significant increases in glycerol release and a concomitant significant decrease of adiponectin from adipocytes in a dose dependent manner. The effects of combined treatments were much larger than individual concentration for each drug. Both drugs had little or no effect on leptin levels, except for a small decrease with 10 μ M rilpivirine alone or when combined with estradiol. In addition, both drugs evoked small increases in the release of resistin and interleukin-8 with significant values at higher doses compared to untreated adipocytes. When adipocytes were pretreated with BNF, either rilpivirine or, estradiol or when combined evoked a much larger release in glycerol and a much larger decrease in adiponectin compared to the absence of BNF.

The study further investigated the lipodystrophic effects of rilpivirine on adipose cells and its nutritional management using quercetin. The results have shown that rilpivirine exerts lipoatrophic effects on adipose cells by impairing triglyceride accumulation, increasing inflammation, repressing anti-oxidant enzymes and inhibiting the expression of genes controlling adipogenesis (PPAR γ , C/EBP α , SREBP-1c, fatty acid synthase and aP2). A combination of rilpivirine and quercetin shows that quercetin was able to decrease inflammation and restore the levels of anti-oxidant enzymes but failed to overcome the lipoatrophic effects of rilpivirine.

The present study also elucidated the role of 17 β -estradiol on human subcutaneous cells *in vitro* either individually or in combination with quercetin. The results revealed that both estradiol and quercetin can promote leanness in part by reducing adipocyte size through reduced uptake of fatty acids and reduced lipogenesis. Experimental data show a decrease in the expression of adipogenesis target genes suggesting anti-adipogenic as well as anti-lipogenic action of estradiol and quercetin in adipose cells.

In conclusion, the results from this study revealed complex interactions between metabolic and inflammatory pathways during the process of adipogenesis. However, the data further suggests that HIV and obese patients who are under increased risk should seek advice from their physicians to offer differentiated and optimized therapeutic approaches.

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STUDENT DECLARATION FORM

Concurrent registration for two or more academic awards

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

Signature of Candidate :Shalini Behl

Type of Award: Doctor of Philosophy (Ph.D.)

School of Forensic and Applied Sciences

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LIST OF ABBREVIATIONS

Acetyl-CoA carboxylase (ACC)
Adipocyte binding protein- 2 (aP2)
Adipocyte determination and differentiation-dependent factor 1 (ADDDF-1)
Adipose most abundant gene transcript 1 (APM1)
American Dietetic Association (ADA)
Anti-retroviral therapy (ART)
Anti-retroviral (ARV)
Aryl hydrocarbon receptor (AhR)
Basic helix-loop-helix (bHLH)
BCA (Bicinchoninic Acid)
Body mass index (BMI)
Bovine Serum Albumin (BSA)
Brown adipose tissue (BAT)
CCAAT/enhancer binding proteins (C/EBPs)
Cyclic adenosine monophosphate (cAMP)
Cyclooxygenase (COX)
Diabetes Mellitus (DM)
DiMethyl Sulphoxide (DMSO)
5, 5'-Dithiobis (2-nitrobenzoic acid) [DTNB]
Dulbecco's modified Eagle's medium (DMEM)
Dulbecco's phosphate buffer saline (DPBS)
Endothelial cell dysfunction (ECD)
Enzyme linked Immunosorbant Assay (ELISA)
17 alpha-estradiol (17 α -E2)
17 beta-estradiol (17 β -E2)
Estrogen Receptor (ER)
Ethylenediaminetetraacetic acid (EDTA)
Fatty acid synthase (FAS)

Fetal Bovine Serum (FBS)
Food and Drug Administration (FDA)
Gelatin-binding protein 28 (GBP28)
Glutathione (GSH)
G Protein-coupled Estrogen Receptor (GPER)
Glutathione peroxidase (GPX)
Glutathione (GSH)
Glycerol-3 phosphate dehydrogenase (GPDH)
High-density lipoprotein (HDL)
Highly active antiretroviral treatment (HAART)
HAART treatment-associated lipodystrophy syndrome (HALS)
Human Immuno Deficiency Virus (HIV)
Impaired glucose tolerance test (IGTT)
Insulin Resistance (IR)
Interleukin-8 (IL-8)
Isobutyl methyl xanthine (IBMX)
Lactate dehydrogenase (LDH)
Lipodystrophy (LD)
Lipoprotein lipase (LPL)
Low-density lipoprotein (LDL)
 β -naphthoflavone (BNF)
Mesenchymal stem cells (MSCs)
Mitotic clonal expansion (MCE)
Natural health product (NHP)
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
Nicotinamide adenine dinucleotide phosphate (NADPH)
Nitric oxide (NO)
Non-steroidal anti-inflammatory drugs (NSAID)
Peroxisome Proliferator-Activated Receptors (PPARs)
Phospholipases (PLA₂s)
Polyacrylamide gel electrophoresis (PAGE)
Polychlorinated biphenyl (PCB)

Prostaglandins (PGs)
Radio-immunoprecipitation assay (RIPA)
Reactive oxygen species (ROS)
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
Simpson Golabi Behmel Syndrome (SGBS)
Single nucleotide polymorphism (SNP)
Sterol regulatory element-binding protein-1 (SREBP-1)
Sterol regulatory element (SRE)
Superoxide dismutase (SOD)
Sulfosalicylic acid (SSA)
Sympathetic nervous system (SNS)
2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)
Thromboxane (TXA₂)
Triglycerides (TGs)
Tumour necrosis factor-alpha (TNF- α)
Ultraviolet (UV)
White adipose tissue (WAT)
World Health Organization (WHO)

CHAPTER 1

Introduction and Background

1. INTRODUCTION AND BACKGROUND

1.1 Obesity: Epidemiology and Patho-physiology

The World Health Organization (WHO) defines obesity as “an abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired”. The currently accepted classification of obesity for epidemiological purposes defines overweight at body mass index (BMI) levels greater than 25 kg/m² and obesity beginning at BMI of 30 kg/m² (WHO, 2000). It is an exaggeration of normal adiposity and is a central player in the patho-physiology of diabetes mellitus (DM), insulin resistance (IR), dyslipidemia, hypertension, atherosclerosis, coronary heart diseases, stroke, liver disease, sleep apnea, osteoarthritis, and gynecological complications and cancer, specifically breast and colon (Obesity and Initiative, 1998). Due to its dramatic increase in prevalence rates over the last few decades, obesity is becoming a pressing international concern for health care officials worldwide. WHO outlines the severity of the problem in the following global key estimates: In 2014, more than 1.9 billion adults aged 18 years and older were overweight. Of these, over 600 million adults were obese. Overall, about 13% of the world’s adult population (11% of men and 15% of women) were obese in 2014, while 39% of adults aged 18 years and over (38% of men and 40% of women) were overweight. The worldwide prevalence of obesity has more than doubled between 1980 and 2014. An estimated 41 million children under the age of 5 years were overweight or obese in the year 2014 and this is an extreme worry concern for the subjects and Governments of the world (WHO, 2016).

Obesity is a multi-factorial disorder resulting from a combination of several lifestyles, environmental and genetic factors. Reduction in physical activity, metabolic rate and thermogenesis eventually decrease energy expenditure leading to increased energy storage and obesity. Some of the causes of obesity include availability of palatable fast food, sedentary lifestyle, certain medications as well as genetic factors. Figure 1.1 illustrates some of the factors that contribute to obesity.

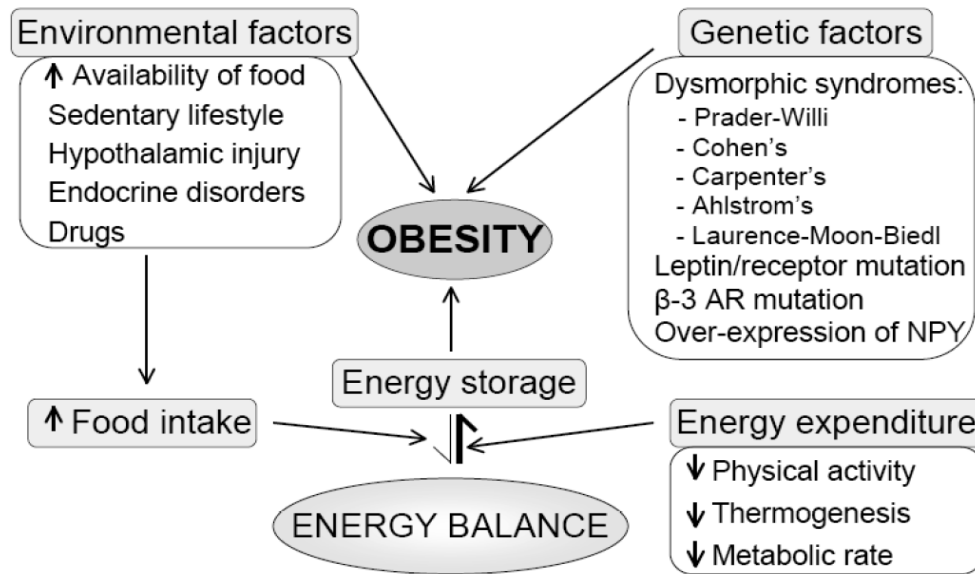


Figure 1.1: Flow diagram showing the energy balance and etiology of obesity (Taken from Gurevich-Panigrahi et al., 2009).

Obesity is characterized by excess fat in the body, contributing considerably to harmful metabolic consequences. Moreover, obesity represents significant medical and socio-economic burden in the world today. According to a recent report, the global cost of treating obesity has risen to a dangerous \$2 trillion USAD (McKinsey Global Institute, 2014).

It is a consequence of adverse increase in adipose tissue mass resulting from increased fat cell size (hypertrophy) or number (hyperplasia) (de Ferranti et al., 2008; Spalding et al., 2008). These two events, together, orchestrate a series of co-morbidities leading to the development of obesity. Targeting adipocyte biology and identifying potential factors that can regulate these processes may be of significant importance in the prevention and treatment of obesity.

1.2 Drug-induced metabolic alterations

Both weight gain and loss are common, but often overlooked side-effects of many widely used drugs. In susceptible individuals, the weight gain may result in clinically significant obesity and associated co-morbidities. Many prescription medications are prominently cited for producing persistent and problematic body weight alterations in treated patients and have a serious impact on medication compliance to an otherwise beneficial treatment. For some (e.g. certain serotonin re-uptake inhibitors, oral contraceptives), the evidence of an effect on body weight is

less consistent (Keith et al., 2006) while others are consistently associated with weight gain, and are considered obesogenic (Pijl et al., 1996; Lean, 2003). This effect can arise as a consequence of differing mechanisms, such as increased appetite (corticosteroids) or reduced metabolic rate (beta-adrenoceptor blockers). In one previous research study, a total of 9% of adults attributed to weight gain due to intake of drugs they were prescribed (Leslie et al., 2014). Table 1.1 shows few classes of medications that cause obesity.

Standard Anti-retroviral therapy (ART) consists of the combination of anti-retroviral (ARV) drugs to maximally suppress the Human Immuno Deficiency Virus (HIV) to stop the progression of HIV disease. Although highly active, anti-retroviral drugs slow down the HIV disease progression and help to prevent HIV-related deaths, their prolonged use can lead to several undesirable side effects. One such common adverse effect of anti-retroviral therapy is lipo-dystrophy in which the body's fat stores are re-distributed. This can involve lipo-atrophy, which refers to loss of fat in the face, buttocks, and limbs, and/or lipo-hypotrophy, in which there is accumulation of fat in the breasts, back or stomach region. These may be accompanied by dyslipidemia, IR (Insulin resistance), impaired glucose tolerance test (IGTT), hypertension, endothelial cell dysfunction (ECD), and altered cytokine and adipokine production (Galescu et al., 2013). Estrogens are important regulators of adiposity and may contribute significantly to the process of adipogenesis.

Table 1.1: Table showing major classes of drugs which can cause weight gain (Taken from Breum et al., 2001).

Anti-psychotics	All subgroups
Anti-depressants	Tricyclic anti-depressants, lithium, MAO inhibitors
Anti-convulsants	Valproate, carbamazepine
Anti-migrane and Anti-histaminergic drugs	Cyproheptadine, flunaizine, pizotifen
Anti-diabetic drugs	Sulfonylurea agents, all insulin preparations, glitazones
Glucocorticoids	Pharmacological doses
Beta-blockers	Non-specific, e.g. propranolol
Sex hormones	Estrogen (high dose), megestrol acetate, tamoxifen
Other	Anti-neoplastic agents

This review now focuses on the role of few such drugs as contributors and/or inhibitors of obesity.

1.2.1 Caffeine

Caffeine (1, 3, 7-trimethylxanthine) is a plant alkaloid found in coffee, tea, chocolate, cola, and soft drinks. It is one of the most commonly consumed ingredient around the world, especially in Europe and North America (Putnam et al., 2006). Interestingly, caffeine has been proposed for weight loss and maintenance for a long time, and is a non-selective antagonist for adenosine receptors (Jacobson, 2009). A number of studies have shown that caffeine reduces body weight and adipose tissue weight in animal models (Yun et al., 2008; Hursel et al., 2010). Caffeine has been reported to have thermogenic effects and can stimulate fat oxidation in vitro, in part through sympathetic activation of the central nervous system (Dulloo et al., 1992). In humans, caffeine has been shown to stimulate thermogenesis and fat oxidation (Dulloo et al., 1989; Astrup et al., 1990; Bracco et al., 1995). It affects the thermogenesis process by inhibiting the enzyme phosphodiesterase. This enzyme degrades intracellular cyclic amino mono phosphate (Cornelis et al., 2007). Usually it hydrolyses cyclic adenosine monophosphate (cAMP) to AMP, but after consumption of caffeine, cAMP concentration rises resulting in increased activity of sympathetic nervous system (SNS). This in turn activates the inactive hormone-sensitive lipase enzyme, which promotes lipolysis (Acheson et al., 2004). Besides the inhibition of phosphodiesterase, caffeine is also known to affect thermogenesis through the stimulation of substrate cycles such as the Cori-cycle and the Free Fatty Acid-triglyceride cycle (Astrup et al., 1990). In the Cori cycle, lactate moves from the muscles to the liver, where it is converted into pyruvate. This pyruvate is then converted to glucose by the enzyme lactate dehydrogenase and circulated back to the muscles through the blood. It has reported that free fatty acid turnover and lipid oxidation are increased after the consumption of caffeine (Astrup et al., 1990). The study also reported that caffeine antagonizes the inhibitory effects of adenosine on lipolysis by adenylyl cyclase. Previously conducted studies have shown that caffeine suppresses intracellular lipid accumulation in 3T3-L1 mature adipocytes (Nakabayashi et al., 2009) and inhibits insulin-stimulated glucose uptake but increases lipolytic effects in adipose cells (Akiba et al., 2004). It is further reported that caffeine significantly stimulates lipolysis in

mature adipocytes (Acheson et al., 2004). Figure 1.2 shows the proposed mechanism of caffeine as inhibitor of adipogenesis in obesity. However, the effects of caffeine on adipogenesis and the process of preadipocyte differentiation into adipocytes have not been determined yet.

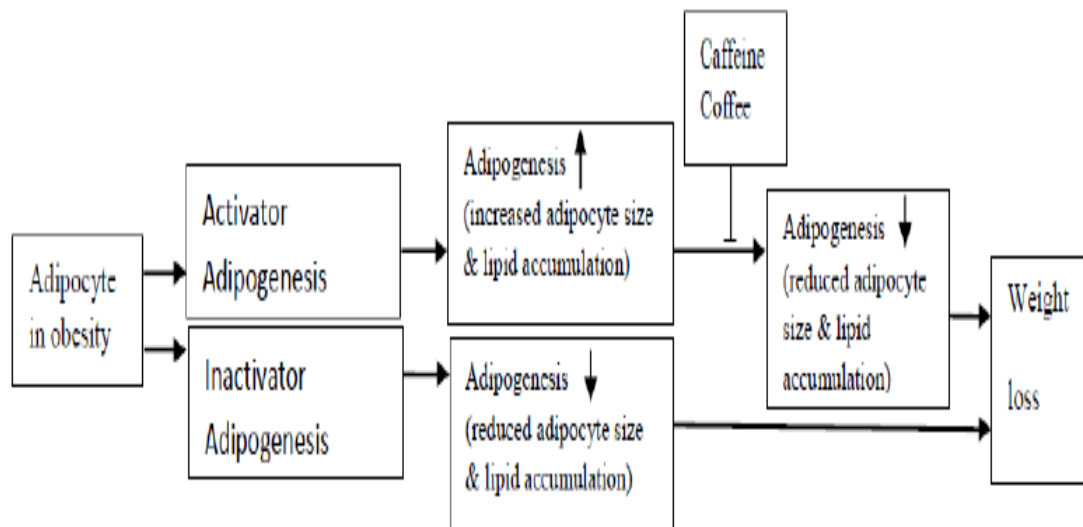


Figure 1.2: Proposed mechanism of caffeine as inhibitor of adipogenesis in obesity (Taken from Ileyana, 2017).

1.2.2 Paracetamol

Paracetamol/Acetaminophen is one of the most popular and commonly used analgesic and antipyretic drugs around the world. It is available without a prescription, both in mono- and multi-component preparations. It is a drug of choice in patients that cannot be treated with non-steroidal anti-inflammatory drugs (NSAID), such as people with bronchial asthma, peptic ulcer disease, hemophilia, salicylate-sensitized people, children under 12 years of age, pregnant or breastfeeding women (Leung, 2012). The mechanism of action is complex and includes the effects of both the peripheral (COX inhibition), and central (COX, serotonergic descending neuronal pathway, L-arginine/NO pathway, cannabinoid system) and redox mechanism (Sharma et al., 2014; Benista et al., 2014). It has been assumed that paracetamol probably acts through the cyclooxygenase (COX) pathway (See figure 1.3). This is the pathway through which the non-steroidal anti-inflammatory drugs (NSAIDs) act. The NSAIDs inhibit production of prostaglandins (pro-inflammatory chemicals; PGE_2 , PGI_2 , $\text{PGF}_{2\alpha}$) and exert consequent effect. They also

influence thromboxane (TXA₂). TXA₂ is a vasoconstrictor, potent hypertensive agent, and facilitator of platelet aggregation.

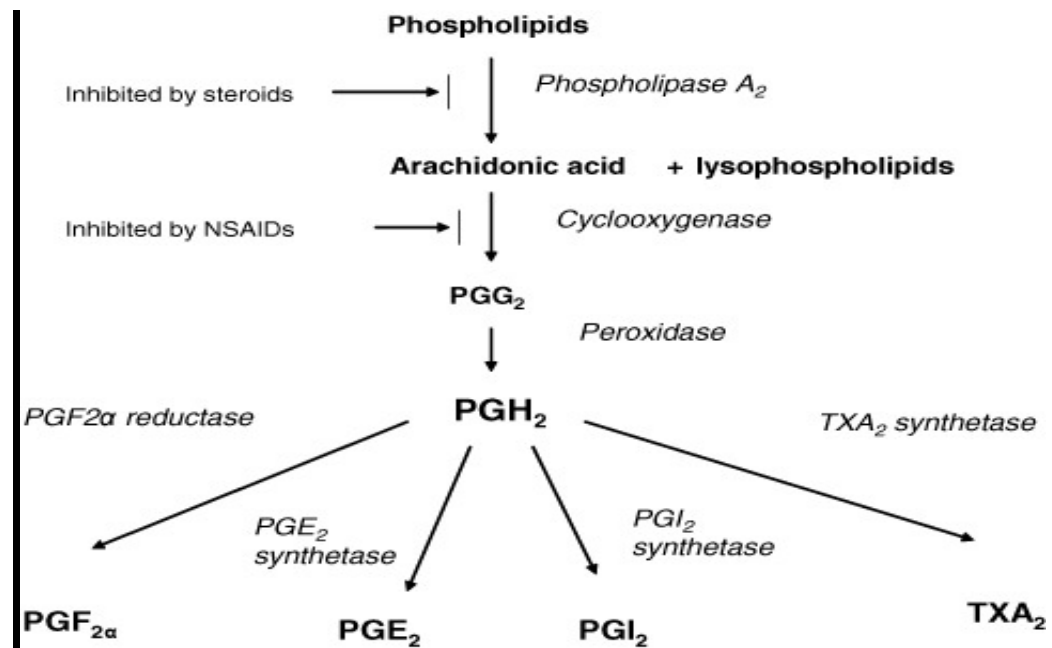


Figure 1.3: Schematic diagram of arachidonic acid metabolism (Taken from Zealand, 2008).

The role of prostaglandins (PGs) and subsequent hormones and enzymes synthesized is essential in adipocyte differentiation. PGs are lipid mediators that undergo synthesis through the action of different enzymes. Archadionic acid is first released by phospholipases (PLA₂s) and undergoes conversion to PGH₂ by one of the two COX isozymes (COX-1 or COX-2). The PGH₂ that is produced is metabolized into different PGs that are involved in adipocyte differentiation which causes the activation of PPAR_γ (Ghoshal et al., 2011; Ueno et al., 2011). Therefore, bearing this expression mechanism in mind- the inhibition of COX-2 would prevent or decrease the conversion of archadionic acid to PGH₂ which will have the ultimate effect of reducing and/or inhibiting adipocyte differentiation. COX-2 activation has also been found to be significantly involved in the development of an inflammatory response in adipose tissue (Hsieh et al., 2010) and has been linked to contribute in brown adipocyte differentiation in mice (Ghoshal et al., 2011).

Despite this proposed mechanism, no conclusive research has yet provided a definitive answer to the role of COX-2 inhibition or over-expression in adipocyte expression. Studies conducted on

3T3-L1 cell lines showed that different adipocyte specific markers which included PPAR γ and adiponectin were found to be decreased after the use of COX inhibitors (Mazid et al., 2006). COX-2 deficient murine models which were supplied with a high fat diet showed lower body mass than their wild type counterparts along with higher metabolic activity, lower PPAR γ , lower inflammatory markers and significantly higher levels of adiponectin (Ghoshal et al., 2011). These variables coincide with other results linking them to increased lipolysis and lower body fat and basal metabolic index (BMI), thus suggesting that the use of a COX-2 inhibitor (such as paracetamol) can provide a protective treatment opportunity against obesity and can be employed as a future anti-obesity medication.

1.2.3 β -naphthoflavone (BNF)

Humans are indeed exposed to an increasing number of environmental xenobiotics. Organic pollutants such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), β -naphthoflavone (BNF), and polychlorinated biphenyl (PCB) are well-known environmental toxins that might affect our endocrine system. Such pollutants are broadly termed as endocrine disruptors. Figure 1.4 illustrates the sources of many common endocrine disruptors in the environment.

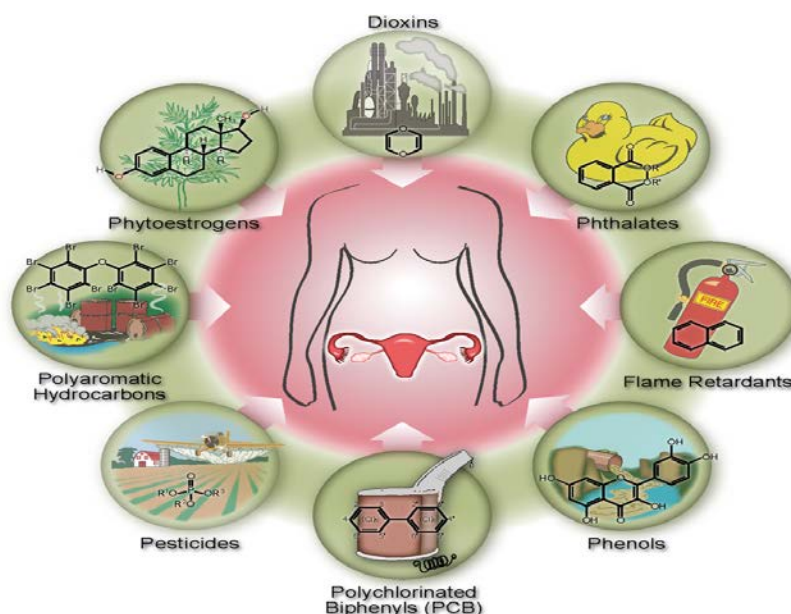


Figure 1.4: Common sources of endocrine disruptors in the environment (Taken from Grindler et al., 2005).

Some of these toxins escape from the body metabolic system and tend to accumulate in the white adipose tissue (WAT). Although mechanisms of this accumulation are still poorly understood, they may modulate the WAT metabolism and function. This could affect the physiological role of WAT, leading, for instance, to the development of obesity related disorders. The mechanisms through which such chemicals modulate the differentiation, metabolism, and secretory function of adipocytes are numerous (Müllerová et al., 2007). The link between exposure to endocrine disruptor xenobiotics and obesity has already been proposed (Elobeid et al., 2008) and some phthalates, which are highly prevalent lipophilic pollutants, have been shown to promote adipocyte differentiation, therefore supporting this hypothesis (Feige et al., 2007). Several epidemiological studies have also linked dioxin exposition to increased risk of diabetes or modified glucose metabolism (Uemura et al., 2008). Although the liver is the main organ involved in metabolizing drugs, the expression and induction of drug-metabolizing enzymes by xenobiotics is also often observed in extra hepatic tissues. It has been reported that rodent adipose tissue expresses functional Aryl hydrocarbon receptor (AhR) and NF-E2-related factor 2 (Yoshinari et al., 2004, 2006). AhR is a cytosolic ligand-activated transcription factor that participates in a variety of metabolic processes, including detoxification and adipogenesis. It would therefore be significant to employ in vitro models to investigate and ascertain the bio-activation potential of such environmental contaminants acting as endocrine disruptors.

1.2.4 Rilpivirine

Rilpivirine (Trade name: Edurant), is a prescription medicine approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV infection in adults and children 12 years of age and older who have never taken HIV medicines before and who have a viral load (number of HIV RNA copies /mL of blood) of 100,000 copies/mL or less at the start of treatment. It belongs to a class (group) of HIV drugs called non-nucleoside reverse transcriptase inhibitors (NNRTIs).

Re-distribution and or accumulation of body fat, including central obesity, dorso-cervical fat enlargement (buffalo hump), peripheral wasting, facial wasting, breast enlargement, and "cushingoid appearance" have been observed in patients receiving antiretroviral therapy. The mechanism(s) and long-term consequences of these events are currently unknown. A causal relationship has not been established. Since rilpivirine is a newly developed medicine, its role in body fat redistribution is still in its infancy stages of investigation. Not much clinical data are available showing its effect on the adipose tissue distribution and function. Clinical trials with

patients taking rilpivirine, in association with distinct drug backbones and in comparison with one of the most commonly prescribed anti-retroviral drug, efavirenz, concluded that both of these drugs had similar antiviral efficacy (Cohen et al., 2011; Molina et al., 2011). However, treatment with rilpivirine caused a much lower increase in blood triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol compared to efavirenz treatment (Cohen et al., 2011; Molina et al., 2011; Wilkin et al., 2012). Another report indicated that there was no change in limb fat in patients taking either rilpivirine or efavirenz in a clinical trial setting (Tebas et al., 2014). Although the results from these clinical trials are contradictory to each other, the approval document of rilpivirine issued in 2011 by the U.S. Food and Drug Administration (FDA) included fat re-distribution among its warnings and precautions. Figure 1.5 shows the factors that might contribute to lipodystrophic effects of these drugs.

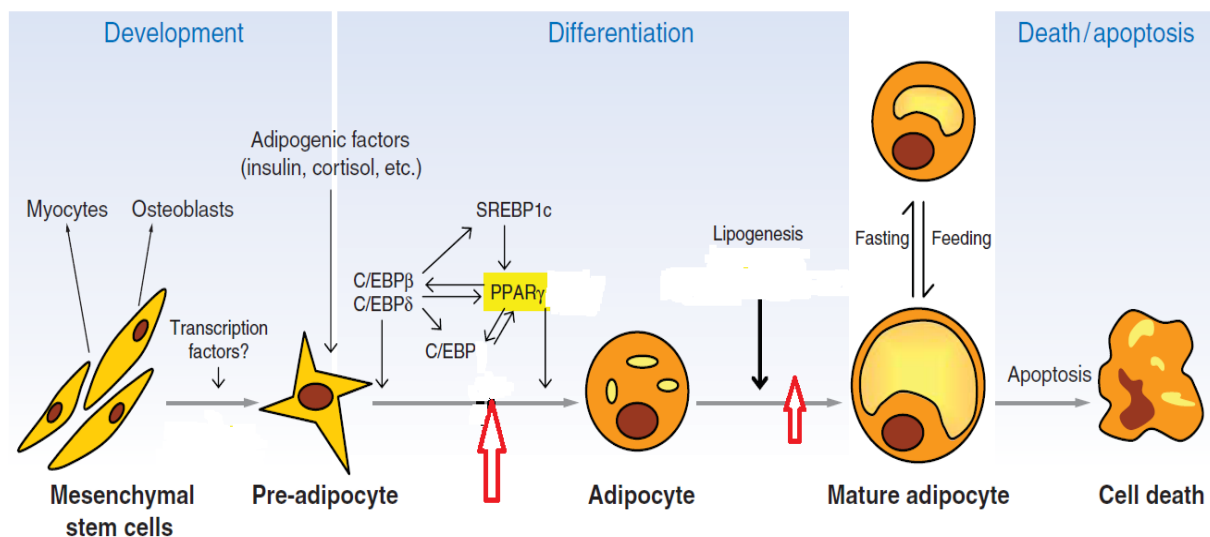


Figure 1.5: Pathways involved in the development, differentiation, and death of adipocytes. Red arrows indicate the factors that might contribute to the lipo-dystrophic effects of anti-retroviral drugs (Modified from Agarwal et al., 2006).

1.2.5 17 β -Estradiol

Estradiol (also known as E2 or 17 β -estradiol) is a naturally occurring hormone that circulates endogenously within the human body. It is the most potent form of mammalian estrogenic steroids and acts as the major female sex hormone. As such, estradiol plays an essential role in

the regulation of the menstrual cycle, in the development of puberty and secondary female sex characteristics, as well as in ageing and several hormonally-mediated disease states. Estrogen mediates its effects across the body through potent agonistic effect of the Estrogen Receptor (ER), which is located in various tissues including in the breasts, uterus, ovaries, skin, prostate, bone, fat, and brain. Estradiol binds to both subtypes of the Estrogen Receptors namely estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Estradiol also acts as a potent agonist of G Protein-coupled Estrogen Receptor (GPER), which has recently been recognized as a major mediator of estradiol's rapid cellular effects (Prossnitz et al., 2014). Figure 1.6 shows the chemical structure of 17 β -estradiol.

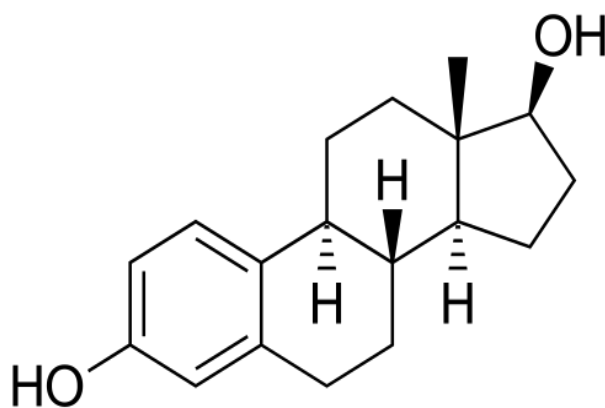


Figure 1.6: Chemical structure of Estradiol (Taken from National centre for biotechnology information, 2016).

Synthetic estrogens are used as part of some oral contraceptives, in estrogen replacement therapy for postmenopausal women, and in hormone replacement therapy for women (Christin-Maitre, 2017). The steroid 17 β -estradiol is the most potent and prevalent endogenous estrogen, although several metabolites of estradiol also have estrogenic hormonal activity (Evans et al., 2015). It is important in the regulation of the estrous and menstrual female reproductive cycles and for the development and maintenance of female reproductive tissues (Ryan, 1982). Estradiol acts primarily as an agonist of the estrogen receptor (ER) which is a nuclear steroid hormone receptor. There are two subtypes of the ER, ER alpha (ER α) and beta (ER β), and estradiol potently binds to and activates both receptors. The result of ER activation is a modulation of gene transcription and expression in ER-expressing cells, which is the predominant mechanism by which estradiol mediates its biological effects in the body. Estradiol also acts as an agonist of membrane estrogen receptors, such as G protein-coupled estrogen

receptor 1 (GPER), a recently discovered non-nuclear receptor for estradiol, via which it can mediate a variety of rapid, non-genomic effects (Nilsson et al., 2011; Prossnitz et al., 2014).

Several studies have provided evidence of a hormonal regulation of pre-adipocyte proliferation (Hausman et al., 2001; Thorn et al., 2007). Estrogens promote subcutaneous fat and regulate adipose tissue distribution through its receptors, ER α and ER β (Matelski., 1985; Kuiper et al., 1996). However, only ER α has been reported to have any major influence on energy homeostasis (Heine et al., 2000) and is necessary for genomic actions of estradiol on body weight regulation (Musatov et al., 2007). 17 β -estradiol has been shown to have stimulatory effects on proliferation of female rat pre-adipocytes (Dieudonne et al., 2000) and in human pre-adipocytes (Anderson et al., 2001).

1.3 Role of adipose tissue in the development of obesity

1.3.1 Adipogenesis

Adipose tissue is an areolar connective tissue that regulates and maintains body temperature, attaches the skin, and shields internal organs. It is further sub-divided into two types namely brown adipose tissue (BAT) and white adipose tissue (WAT). Figure 1.7 shows the distribution of white and brown fat in the human body.

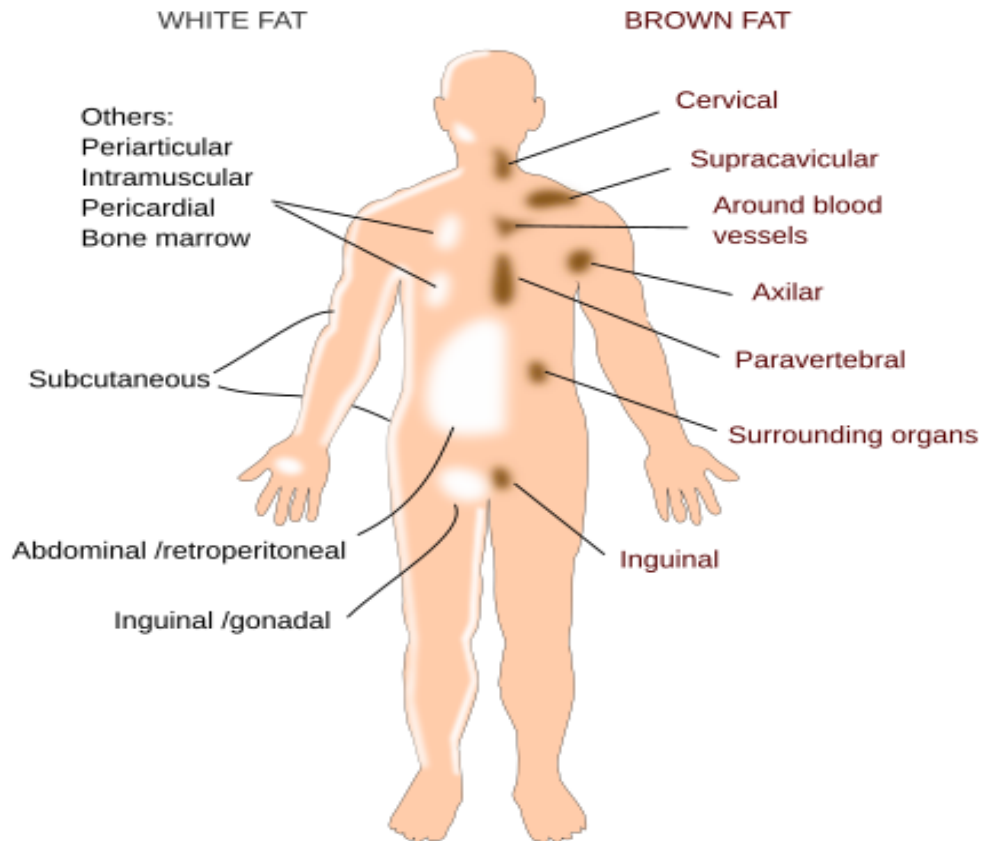


Figure 1.7: Distribution of white and brown fat in the human body (Modified from Gesta et al., 2007).

While WAT serves as the reservoir of energy, where triglycerides are stored in large lipid droplets, BAT mainly serves in the regulation of body temperature and energy expenditure (Girard et al., 2008). It is now a well-accepted fact that mesenchymal stem cells (MSCs) act as precursor cells to proliferate and undergo differentiation to become mature adipocytes. The process of differentiation of the fibroblast like pre-adipocytes into mature lipid-laden, insulin-responsive adipocytes is known as adipogenesis (Lefterova et al., 2009).

1.3.2 The Adipocyte life cycle

The adipocyte life cycle starts with differentiation of adipocytes from either committed embryonic stem cells or mesenchymal stem cells. This includes a growth phase followed by growth arrest, clonal expansion, and a complex sequence of changes in gene expression leading

to storage of lipid and finally death (Gregoire, 2001). Figure 1.8 shows the phases in life cycle of an adipocyte.

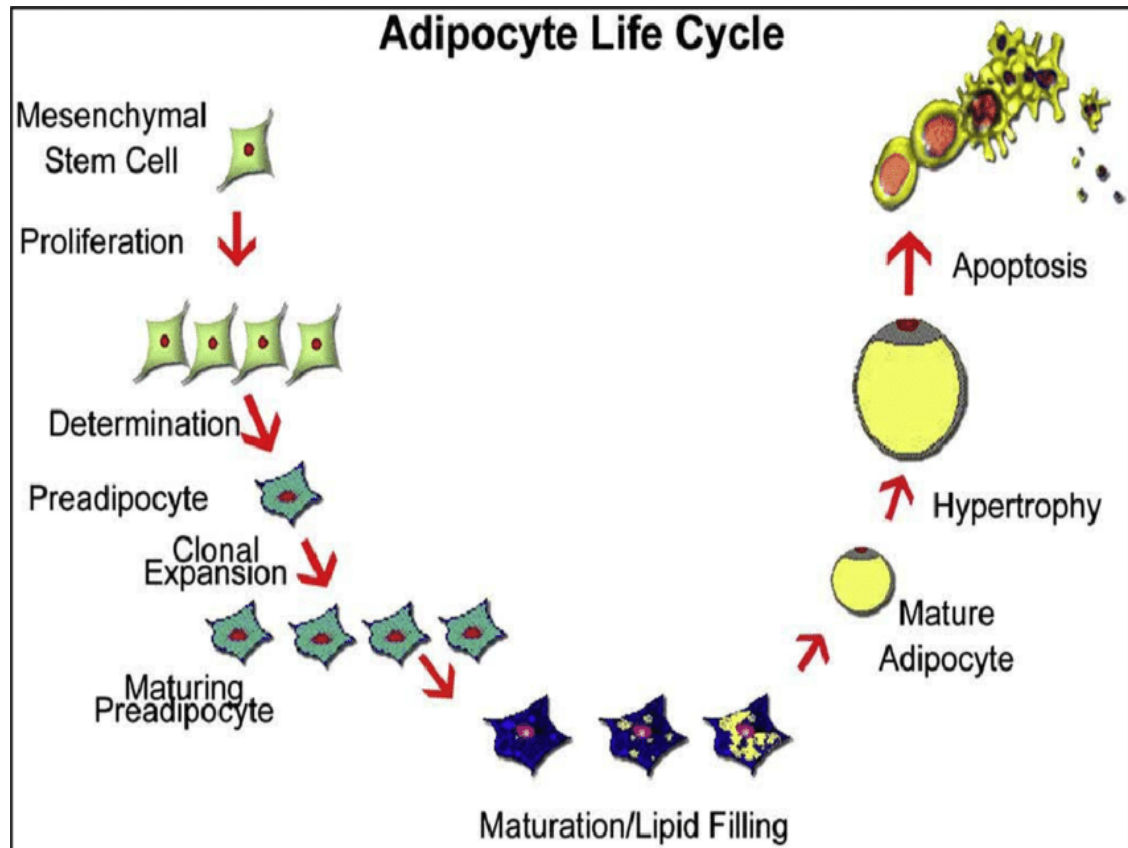


Figure 1.8: Flow diagram showing the life cycle of an adipocyte. Note the different phases involved in the differentiation of mesenchymal stem cells into mature adipocytes (Taken from (Abdel-sattar et al., 2014)).

Once triggered to mature, pre-adipocytes proliferate and undergo growth arrest followed by a round of cell division known as clonal expansion. These cells then commit to differentiate into mature adipocytes. This process is accompanied by a dramatic increase in expression of adipocyte-specific genes. Several adipokines, hormones, enzymes, nutritional and environmental factors influence adipocyte life cycle contributing to overall adipose tissue growth and development.

1.4 Inflammatory markers of obesity

Over the recent years, an understanding of the adipocytes as mere fat storage cells has changed dramatically. They are now known to participate in several metabolic regulations by secreting a

large number of protein mediators that are termed adipocytokines /adipokines. Research studies are now concentrating on the concept that obesity could be a source of inflammatory factors, which enhance insulin resistance and create a chronic low-grade systematic inflammation (Kang et al., 2016). Some of the well-studied adipokines that influence adipocyte growth through both central and peripheral mechanisms include adiponectin, plasminogen activator inhibitor-1, tumour necrosis factor-alpha (TNF- α), leptin, resistin, interleukin-6 and macrophage chemoattractant protein-1. These are implicated for their role in different mechanisms like regulation of energy balance, insulin action glucose metabolism, inflammation and immunity (Rondinone, 2006). Figure 1.9 shows the contribution of some of the important adipokines in the development of obesity and its related complications.

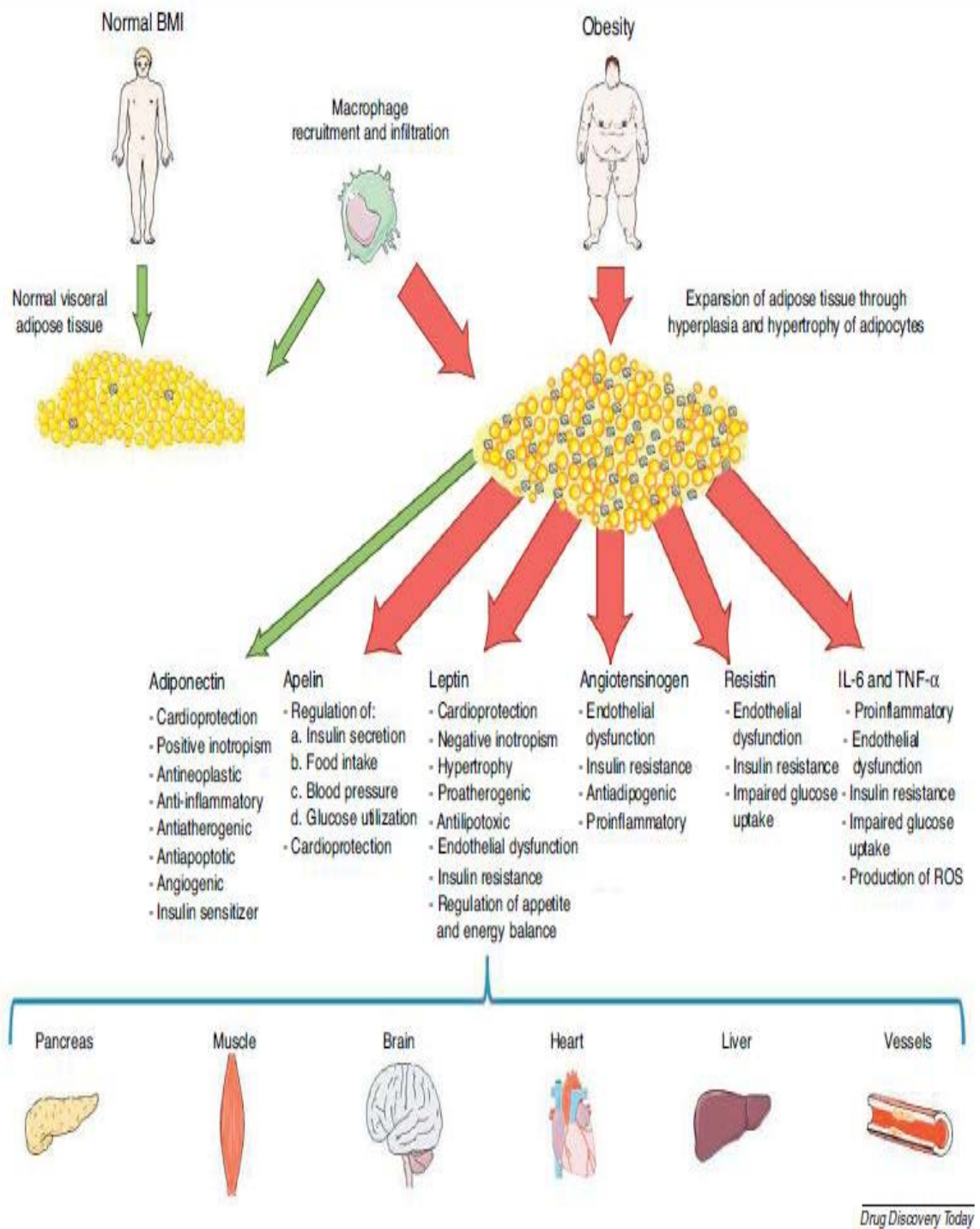


Figure 1.9: Diagram showing the contribution of adipokines to obesity and metabolic syndrome abnormalities (Taken from Falcão-Pires et al., 2012).

1.4.1 Leptin

Leptin is a 16-kDa polypeptide containing 167 amino acids. Moreover, it was originally discovered as the missing protein in the genetically obese *ob/ob* mouse (Halaas et al., 1995) and was later widely recognized for its ability to regulate adipose tissue mass by influencing food intake and energy expenditure (Brennan et al., 2006). Leptin concentration in plasma is proportional to total body adiposity and direct nutritional state (Frederich et al., 1996). It is present in human serum in ranges of 1-15 ng/ml in non-obese individuals, and levels more than 30 ng/ml in individuals with BMI ≥ 30 kg/m² (Caro et al., 1996).

The role of leptin in counteracting obesity is mediated through its receptors, which are members of the cytokine receptor class I super-family and are expressed in both the central nervous system and periphery (Elmquist et al., 1999). These receptors orchestrate complex metabolic changes in a number of organs and tissues, altering nutrient flux to favor lipid mobilization over lipid storage (Baile et al., 2000). Leptin receptors are highly expressed in hypothalamic neurons involved in regulation of food intake and they are probably the most physiologically relevant target for leptin especially since brain specific deletion of leptin receptor is sufficient to cause severe obesity (Cohen et al., 2001).

Previously documented evidences on the effects of leptin on energy homeostasis are available and previous studies have suggested that leptin can also decrease adiposity by triggering apoptosis and lipolysis in adipocytes (Della-Fera et al., 2008). Adipose tissue from rats that were given cerebral ventricular injections of leptin demonstrated features of apoptosis, including inter-nucleosomal fragmentation of genomic DNA, elevated levels of DNA strand breaks, reduction in total DNA content, and cellular volume (Qian et al., 1998). The mechanisms of leptin-induced adipose tissue apoptosis are not fully understood. Most of the effects of leptin on adipocyte apoptosis and lipolysis are believed to be centrally-mediated by stimulation of the sympathetic nervous system. The possibility that leptin can act directly on adipocytes to induce apoptosis has not been thoroughly investigated so far. Although it has been reported that leptin did not directly stimulate apoptosis in 3T3-L1 adipocytes in vitro (Ambati et al., 2007), it significantly reduced lipid accumulation and GPDH (Glycerol-3 phosphate dehydrogenase) activity in maturing 3T3-L1 pre-adipocytes, indicating that leptin may not act directly to induce adipocyte apoptosis, but can act directly to inhibit maturation of pre-adipocytes. Figure 1.10 shows the three general ways in which alterations of the leptin regulatory loop could lead to

obesity. a). Failure to produce leptin, as occurs in ob/ob mice. b). Inappropriately low levels of leptin secretion for a given fat mass. In the latter case, the fat mass would expand until 'normal' leptin levels are reached, resulting in obesity. c). Finally, obesity could result from either relative or absolute insensitivity to leptin at its site of action. Such resistance would be associated with increased circulating leptin, analogous to the increased insulin levels seen with insulin-resistant diabetes. In general, high plasma leptin levels are evident in obese mice and rodents. Differences in leptin production and leptin sensitivity could be the result of genetic, environmental and psychological factors.

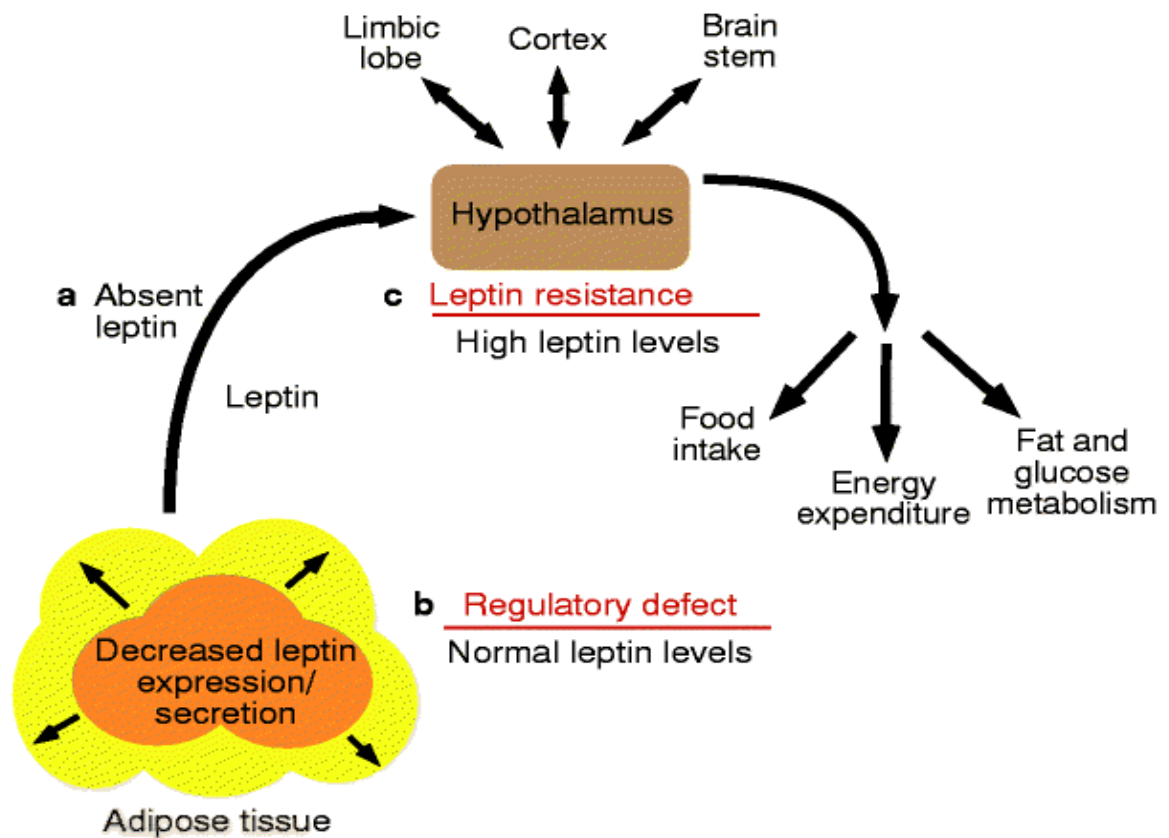


Figure 1.10: Flow diagram showing general ways in which dysregulation in leptin production may lead to obesity (Taken from Friedman et al., 1998).

ARVs, such as nevirapine and efavirez, have been reported to reduce leptin expression to a significant level in human adipocytes (Díaz-Delfín et al., 2011; 2012). Another ARV, ritonavir, also decreased leptin levels in mice (Vyas et al., 2010). An investigational study on serum leptin concentrations in women taking oral contraceptives containing the same gestagen and different low-doses of ethinyl estradiol, did not exert any influence on serum leptin or BMI, and therefore did not induce any significant influence on body energy metabolism (Rechberger et al., 1999).

1.4.2 Adiponectin

Adiponectin, having been discovered by several groups, has been attributed numerous different names including ACRP30 (adipocyte complement-related protein of 30 kDa) or adipoQ in mouse and GBP28 (gelatin-binding protein 28) or APM1 (adipose most abundant gene transcript 1) in humans (Kadowaki et al., 2005). It is specifically expressed in differentiated adipocytes, and its expression is higher in subcutaneous than visceral adipose tissue (Fain et al., 2004). Plasma levels of adiponectin, which constitute 0.01 % of circulating proteins, are between 5 to 30 mg/L in lean control subjects (Lihn et al., 2004). Moreover, a negative correlation between plasma concentration of adiponectin and BMI in men and women ($r = -0.71$; $P < 0.0001$; and $r = -0.51$, $P < 0.0001$, respectively) has been reported in the literature (Arita, 2012). Data from both animal and human studies indicate that adiponectin has insulin-enhancing as well as anti-inflammatory actions (Kadowaki et al., 2006). Figure 1.11 shows some of the anti-inflammatory effects of adiponectin. Adiponectin levels are markedly reduced in obese and diabetic mice, and injection of the adiponectin globular domain to these animals ameliorates insulin resistance, an effect that can be ascribed to an enhancement of fatty acid- β oxidation in skeletal muscle and a decrease of hepatic gluconeogenesis mediated by AMP-activated protein kinase (Berg et al., 2001; Maeda et al., 2001; Yamauchi et al., 2002; Combs et al., 2004). Furthermore, adiponectin knockout mice showed increased susceptibility to diet-induced insulin resistance as well as injury-induced arterial stenosis and neointimal formation (Kubota *et al.*, 2006; Matsuda and Shimomura, 2013). Consistent with the results from animal models, several cross-sectional studies have shown an association between low serum adiponectin levels and insulin resistance, type 2 diabetes (T2DM) and cardiovascular diseases (CVDs) in humans (Hotta et al., 2001; Spranger et al., 2003; Yang et al., 2005).

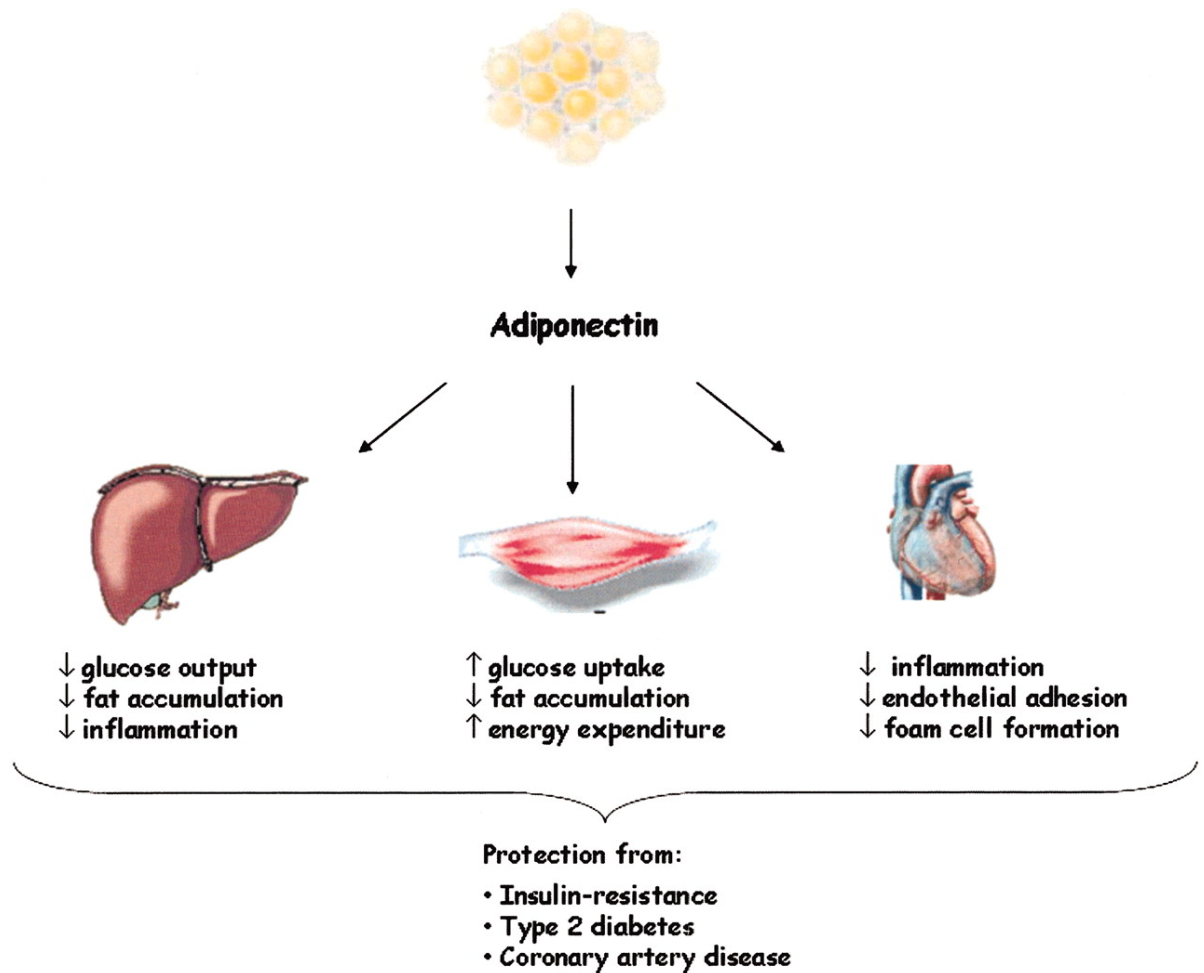


Figure 1.11: Diagram showing the proposed salutary effects of adiponectin (Taken from Menzaghi et al., 2007).

A study on Pima Indian population showed that individuals with low concentrations of plasma adiponectin have reduced insulin sensitivity, and they tend to develop T2DM (Lindsay et al., 2002). Elevated plasma adiponectin levels were found in low BMI diabetic and non-diabetic subjects.

Adiponectin exerts its biological effects by binding to ubiquitously expressed receptors AdipoR1 and adipoR2, as well as Y-cadherin. The synthesis and secretion of adiponectin are regulated by endoplasmic reticulum molecules including calcium (Ca^{2+}), and once in circulation, its stability is maintained by post –transcriptional modifications (Caselli, 2014).

Besides the metabolic effects, adiponectin executes anti-inflammatory effects through inhibition of NF- κ B signaling and reduced secretion of several inflammatory cytokines released from monocytes, macrophages and dendritic cells (Scherer, 2006).

In vitro studies in 3T3-L1 and Simpson Golabi Behmel Syndrome (SGBS) adipocytes treated with ARVs have shown inconsistent results. For example, ritanovir, in particular, has been shown to reduce adiponectin mRNA expression and its secretion by up to 50% as compared to control (Grigem et al., 2005; Lagathu et al., 2007; Adler-Wailes et al., 2012; Kim et al., 2012). Moreover, it has been suggested to occur as a result of increased oxidative stress (Lagathu et al., 2007) and also by a negative feedback mechanism involving inflammatory cytokines (Luo et al., 2009). However, in other studies ARVs either increase adiponectin expression (Díaz-Delfín et al., 2011), or have no effect (Jones et al., 2008). The use of stavudine was associated with low adiponectin level in plasma and increased insulin resistance compared with abacavir (Podzamczar et al., 2007).

Currently, little is known about the association of adiponectin and female sex steroids. A cross-sectional study with 32 healthy women showed that total adiponectin, high molecular weight multimer, and medium molecular weight hexamer were inversely related to estradiol and progesterone. However, a low molecular weight trimer was not associated with female sex hormone. The study also indicated that the effect of estradiol and progesterone on insulin function might be mediated indirectly by their interactions with adiponectin (Leung et al., 2009).

1.4.3 Resistin

Resistin is a 12.5 kDa cysteine-rich protein secreted in rodents by adipocytes and in humans by macrophages (Steppan et al., 2001; Park and Ahima, 2013) in response to chronic inflammation, a characteristic feature of obesity and T2DM. Among the adipokines, a characteristic expression profile for resistin was obtained during the differentiation and maturation of 3T3L-1 cells and primary pre-adipocytes obtained from Zucker obese rats (Ikeda et al., 2013).

Plasma resistin levels have been shown to be significantly greater in HIV-infected individuals without lipo-dystrophy (LD) compared with those with lipo-dystrophy and uninfected individuals (4.21 vs. 3.64 vs. 2.74 ng/ml, respectively), and in patients with isolated linolenic acid compared with those with a mixed form of LD (4.64 vs. 3.27 ng/ml) (Escoté et al., 2011). These findings have been supported by another group (Arama et al., 2014). A single nucleotide polymorphism

in the resistin gene was reported to increase the risk of metabolic complications associated with ARVs (Ranade et al., 2008). The degree of involvement and the specific role of resistin in the metabolism of ARVs still remain to be elucidated.

17 β -estradiol has been reported to stimulate resistin gene expression in 3T3-L1 adipocytes via the estrogen receptor extracellularly to regulate kinase enzymes, and CCAAT/enhancer binding protein- pathways (Chen et al., 2006). Despite the importance of resistin, relatively little is known about the control of production of resistin by sexual hormones and still much more needs to be studied.

1.4.4 Interleukin-8 (IL-8)

Interleukin-8 is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells (Hedges et al., 2000). One of the well-known and important complications of obesity is atherosclerosis. Since it is recognized that atherosclerosis might have inflammation as an important part of its pathology, IL-8 has been suggested to contribute to atherogenesis through several actions. Previously reported observations have demonstrated proximity to the initial pre-atherosclerotic lesion as well as in the advanced atherosclerotic plaque. Oxidized low-density lipoprotein, which is well described in the atherosclerotic process, can stimulate the release of IL-8 from macrophages in the atherosclerotic lesion (Liu et al., 1997). Because the adipose tissue can produce and release various cytokines and IL-8, it is possible to extrapolate a link to the atherosclerotic process. It may be of great interest to investigate the ability of human adipose tissue and isolated adipocytes to express and release IL-8. Increased expression of IL-8 was observed in HIV infected people (Lihn et al., 2003).

1.5 Oxidative Stress and Antioxidant defense

In addition to exacerbated inflammatory outcomes, accompanied by infiltration of immune cells in adipocytes, obesity is also characterized by increased oxidative stress. Release of inflammatory adipocytokines and oxidative stress machinery are not only inter-related, but their impairment can lead to pathogenesis of obesity and its associated risk factors. Oxidative stress refers to any disturbance in the balance of antioxidants and pro-oxidants in favour of the later due to different factors such as ageing, drug action and toxicity, inflammation and/or addiction (Sies et al., 1985). It is an imbalance between antioxidants [e.g., superoxide dismutase (SOD) and

glutathione peroxidase (GPX)] and reactive oxygen species [e.g., superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-)] (Sies, 1997). Oxidative stress causes healthy cells of the body to lose their function and structure by attacking them. Damage to DNA, proteins, and other macromolecules due to oxidation has been implicated in the pathogenesis of a wide variety of diseases (Halliwell et al., 1994). Both cell culture and animal studies have demonstrated that oxidative stress can cause an increase in pre-adipocyte proliferation, adipocyte differentiation, and the size of mature adipocytes (Furukawa et al., 2004; Lee et al., 2009; Higuchi et al., 2013). Oxidative stress plays critical roles in the pathogenesis of various diseases (Brownlee, 2001). In the diabetic condition, oxidative stress impairs glucose uptake in muscle and fat (Rudich et al., 1998; Maddux et al., 2001) and decreases insulin secretion from pancreatic β cells (Matsuoka et al., 1997). Increased oxidative stress also underlies the pathophysiology of hypertension (Nakazono et al., 1991) and atherosclerosis (Ohara et al., 1993) by directly affecting vascular wall cells. Figure 1.12 shows a working model of how increased oxidative stress in potentially relates to various obesity-related diseases.

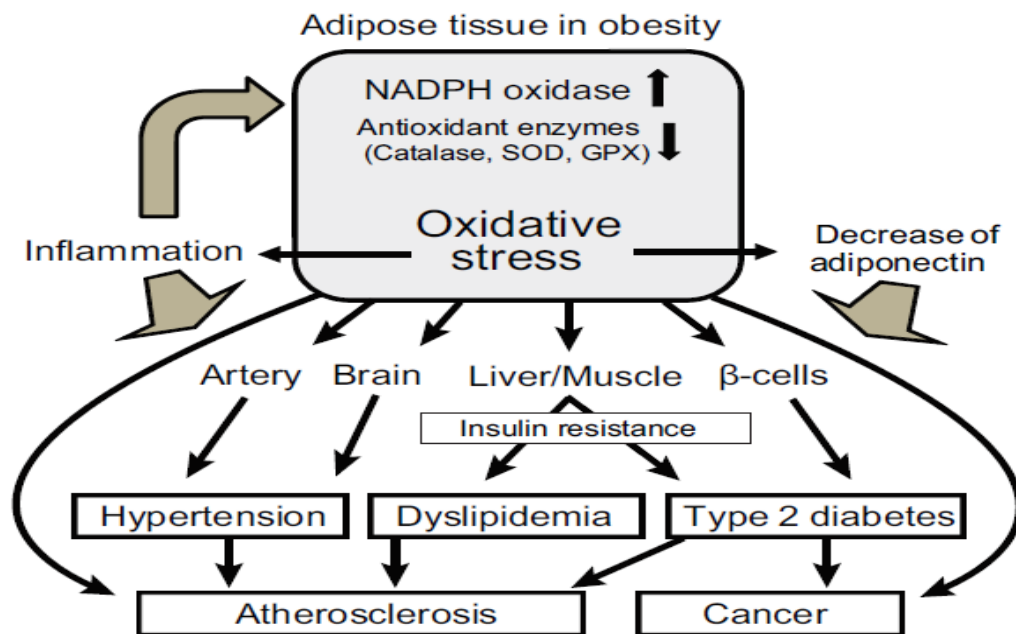


Figure 1.12: Working model illustrating how increased oxidative stress in adipose tissue potentially relates to various obesity-related diseases. Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; GPX, glutathione peroxidase (Taken from Matsuda et al., 2013).

Oxidative stress is controlled by the endogenous antioxidant defense system, which includes antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase; and non-enzymatic compounds such as ferritin, transferrin, bilirubin, ceruloplasmin and even albumin carrier low molecular weight, such as uric acid and lipoic acid (Poljšak et al., 2014). Antioxidants are able to trap free radicals generated by cellular metabolism or exogenous sources through the donation of hydrogen atoms of these molecules, breaking the chain reaction, which prevents attack on lipids, amino acids in proteins, double bond of the polyunsaturated fatty acids, and DNA bases, avoiding formation of lesions and loss of cell integrity (Sies, 1993). Another role of antioxidants is the protection mechanism, which acts in the repair of damage caused by free radicals, a process related to the removal of the DNA molecule of damage and restoration of damaged cell membranes (Sies, 1991).

1.5.1 Biomarkers of oxidative stress

Species or molecules whether endogenous (internally synthesized) or exogenous (consumed), can play a major role in the antioxidant defense and this may be considered as biomarkers of oxidative stress.

1.5.1.1 Catalase

Catalase is an anti-oxidative enzyme which plays an important role against oxidative stress-generated complications such as obesity, diabetes and cardiovascular diseases (Chelikani et al., 2004). It acts as a major regulator of hydrogen peroxide metabolism. Hydrogen peroxide (H_2O_2) is a highly reactive small molecule formed as natural by-product of energy metabolism which in excessive concentration may cause significant damages to proteins, DNA, RNA, and lipids (Takemoto et al., 2009). Catalase catalytically converts H_2O_2 into water and oxygen and thus neutralizes it. Increased risk of obesity and diabetes has been documented in patients with catalase deficiency. In case of catalase deficiency, beta cell of pancreas that contain large amount of mitochondria, undergoes oxidative stress by producing excess reactive oxygen species (ROS) leading to dysfunction of β -cells and ultimately obesity and diabetes (Jamieson et al., 1986).

1.5.1.2 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is the antioxidant enzyme that catalyses the dismutation of superoxide anion ($O_2^{\cdot -}$) into hydrogen peroxide and molecular oxygen (Faraci et al., 2004; Wang et al., 2016). SOD provides first line of defense against ROS mediated cellular and histological damages by facilitating the conversion of superoxide radicals into hydrogen peroxide, and in the presence of other enzymes it is converted into oxygen and water (Tiwari et al., 2013). All mammalian tissues contain three forms of SOD: Cu-Zn-SOD, Mn-SOD, and extracellular EC-SOD, and each of them is a product of a distinct gene (Beyer et al., 1991).

Superoxide reacts rapidly with nitric oxide (NO), reducing NO bioactivity and producing the oxidative peroxynitrite radical (Guzik et al., 2002). SOD, a major defender against superoxide, in the kidneys during the development of murine diabetic nephropathy and down-regulation of renal SOD (SOD 1 and SOD 3) may play a key role in the pathogenesis of diabetic nephropathy (Fujita et al., 2009). Over expression of SOD or the supplements of antioxidants including SOD mimetics, targeted to overcome oxidative stress, reduce ROS, and increase antioxidant enzymes, has been shown to prevent diabetes mellitus (Wang et al., 2011).

1.5.1.3 Glutathione (GSH)

Glutathione (GSH), a tripeptide, γ -L-glutamyl-L-cysteinyl glycine, is present in all mammalian tissues at 1–10 mM concentrations (highest concentration in liver) as the most abundant non-protein thiol that defends against oxidative stress (Lu, 2013). GSH functions by maintaining SH groups of proteins in a reduced state, participate in amino acid transport, detoxify foreign radicals, act as coenzyme in several enzymatic reactions, and also prevent tissue damage (Tsai et al., 2012). GSH is also considered as a biomarker of redox imbalance at cellular level (Rizvi et al., 2011). Both obesity and diabetes have been reported to induce alterations in the activity of enzymes glutathione peroxidase and glutathione reductase. These enzymes metabolize peroxide to water and play a role in converting glutathione sulphide back into glutathione (Maritim et al., 2003). Any alterations in the levels of these enzymes make the cells prone to oxidative stress and hence cellular injury.

1.5.1.4 Nitric Oxide (NO)

Under normal physiological conditions, nitric oxide (NO) is a critical homeostatic regulator of the vessel wall and plays a role in the maintenance of vascular tone and reactivity (Verma et al., 2002). NO acts as a physiological regulator of diverse functions in several tissues including cardiovascular, neuromuscular, neurological, genitourinary, gastrointestinal and renal. Inhibitors of nitric oxide synthase reduce NO production and prevent the decrease in insulin secretion caused by free fatty acids (Shimabukuro et al., 1997). It acts as an important anti-atherogenic agent and inhibits platelet activation and aggregation, leukocyte chemotaxis, and endothelial adhesion (Chakraborty et al., 2003). Under conditions of overweight and obesity, endothelium-dependent vaso-dilation of NO is impaired (DeSouza et al., 2005).

1.6 Transcriptional regulation of adipogenesis

Differentiation of pre-adipocytes into adipocytes involves a comprehensive network including a number of transcription factors responsible for the expression of key proteins that induce mature adipocyte formation. This highly regulated transcriptional cascade begins with re-entry of growth arrested pre-adipocytes into the cell cycle where they undergo several rounds of mitosis. This initial phase is known as mitotic clonal expansion (MCE) and is accompanied by the transient expression of C/EBP- β and C/EBP- δ . These transcription factors subsequently stimulate transcription of PPAR, which in turn can activate C/EBP- α . PPAR- γ and C/EBP- α exist in a positive feedback loop to propagate differentiation and induction of late adipogenic genes including aP2 and Fas in the terminal differentiation phase (Rosen et al., 2000).

1.6.1 Peroxisome Proliferator-Activated Receptors (PPAR)

Peroxisome Proliferator-Activated Receptors (PPARs) are a nuclear hormone receptor super family of ligand-activated transcription factors, which bind to the promoter of target genes leading to increased or decreased DNA transcription upon binding of ligands, and are involved in various biological processes such as energy metabolism, cell proliferation and inflammation (Kersten, 2002). PPAR plays a dominant role in adipogenic differentiation, glucose metabolism, inflammation and other physiological processes, and is also a receptor of an important class of anti-diabetic drugs (Kliwer et al., 1994; Tontonoz and Spiegelman, 2008). A series of gain-of-function studies in which PPAR- γ was ectopically expressed in non-adipogenic mouse fibroblasts showed that PPAR- γ alone can initiate the entire adipogenic program, giving rise to fat cells that

are capable of many of the functions of mature adipocytes (Tontonoz et al., 1994).

1.6.2 CCAAT/Enhancer-Binding Proteins (CEBP)

The CCAAT/enhancer binding proteins (C/EBPs) belong to a family of highly conserved basic leucine zipper transcription factors that play an important role in adipocyte differentiation. C/EBP- α is important in terminal differentiation of adipocytes, as absence of this factor leads to insulin resistance in in vitro experiments and hinders formation of WAT in vivo. In contrast, development of BAT is independent of C/EBP (El-jack et al., 1999; Linhart et al., 2001).

C/EBP- β and C/EBP- δ have been postulated to be the first transcription factors induced during induction of adipogenesis, and therefore, play an important role in directing the differentiation process (Darlington et al., 1998). The importance of C/EBP- β and C/EBP- δ has been demonstrated in loss-of-function and gain-of-function studies where embryonic fibroblasts from mice lacking these two markers were unable to differentiate in response to hormonal induction. Consequently, these cells failed to express other important adipogenic markers such as C/EBP, PPAR or adipocyte binding protein 2 (aP2)/Fatty acid binding protein 4 (FABP4). These results suggest that in vitro, adipocyte differentiation proceeds according to the proposed transcriptional cascade in which C/EBPs and PPAR families of transcriptional factors are activated sequentially leading to formation of mature adipocytes (Tanaka et al., 1997).

1.6.3 Sterol Regulatory Element-Binding Protein -1 (SREBP)

The sterol regulatory element-binding protein-1 (SREBP-1), also referred to as the adipocyte determination and differentiation-dependent factor 1 (ADD1), is a basic helix-loop-helix (bHLH) leucine transcription factor that is associated with adipocyte differentiation and cholesterol homeostasis (Yokoyama et al., 1993). SREBP-1 is expressed in different types of tissues but is predominantly expressed in brown adipose tissue (Tontonoz et al., 1993). As a member of bHLH transcription factor family, SREBP-1 has dual DNA binding specificity, in that it can bind to an E-box motif and a sterol regulatory element (SRE). Thus, when expressed in fibroblasts, SREBP-1 activates transcription through both the E-box motif and SRE, providing a novel mechanism to coordinate different lipid metabolism pathways (Yokoyama et al., 1993; Kim et al., 1995). SREBP-1 plays a role in adipocyte gene expression by regulating the expression of FAS and LPL, important genes involved in fatty acid metabolism (Kim, 1996).

1.6 Potential health benefits of bioactive compounds in combating obesity /Dietary interventions to combat drug induced inflammation and oxidative stress

The popularity of dietary supplements for weight management has increased over the past years and a wide variety of these products are available over the counter. They represent an attractive adjuvant to traditional therapy because most of them have a low toxicity profile and are accessible to general population. It is therefore not surprising that these supplements account for more than \$37 billion in sales in the USA (Bradley et al., 2015). A national survey published in 2008 found that 33.9% of adults who have made a weight loss attempt had used a dietary supplement to do so (Pillitteri et al., 2008). Despite widespread use, there are still limited data on the safety and efficacy of the products currently on the market. Identification of such dietary compounds has prompted researchers to explore the vast array of their beneficial effects. The American Dietetic Association (ADA) acknowledges the significance of consumption of bioactive compounds and therefore supports the marketing of functional foods, either conventional or modified, when there is substantial scientific evidence of benefit (Hasler et al., 2009).

1.6.1 Quercetin

Quercetin a dietary flavonoid is abundantly present in different types of fruits and vegetables (Alinezhad et al., 2013). Onions, red apples, red wine, tea, cranberry, kale, hot peppers, coriander, fennel, radish and broccoli are considered rich sources of quercetin (Aras et al., 2014). Quercetin present in plant species is commonly found in glycoside forms (Rice-Evans, 2001), with rutin being the most common (Manach et al., 1997). Other glycoside forms of quercetin commonly found in the human diet are 3-rutinoside (occurring in black tea), O-glucoside (onions), quercetin galactosides (apples) and quercetin arabinosides (berries) (Hollman et al., 1997).

The anti-inflammatory and anti-oxidative roles of quercetin to ameliorate obesity via different molecular pathways have been widely studied in animal models and cell lines over the past decade. Circumstantial evidences from different research groups in C57BL/6J mice have reported that a high-fat diet containing quercetin (0.05–0.25%) decreased body weight, liver fat accumulation, blood glucose, plasma and liver tri-acylglycerols, improved TBARS and glutathione levels in the liver. In addition, suppressed expression of important genes, including PPAR γ , a transcription factor that regulates high-fat diet-induced fat accumulation in the liver was

observed previously (Jung et al., 2013). Other studies on Zucker obese rats showed that quercetin increased the plasma adiponectin concentration, reduced plasma nitrate and nitrite levels and enhanced visceral adipose tissue endothelial (Rivera et al., 2008). Apart from animal models, cell lines have also been used extensively to understand how quercetin interferes with lipid metabolism to prevent obesity especially at molecular level.

The protective effects of this compound have been linked to its anti-inflammatory and/or antioxidant properties. Anti-inflammatory effects of quercetin have been reported for both adipocytes and macrophages and these activities are mediated by NF- κ B associated mechanisms (Overman et al., 2011). This study by Overman et al in 2011 reported that quercetin may lower inflammation in adipose tissue by reducing the infiltration of macrophages and preventing infiltrated macrophage-mediated inflammation and insulin resistance in human adipocytes and that these effects were associated with quercetin dependent suppression of NF- κ B activation. Quercetin is known to decrease TNF- α -induced NF- κ B transcriptional activity in primary human adipocytes and further attenuates the TNF- α -mediated suppression of PPAR- γ activity and PPAR- γ target genes (Chuang et al., 2010). The anti-obesity action of quercetin is mediated by the AMPK and MAPK signaling pathways. It has been reported to attenuate adipogenesis and decrease expression of adipogenesis-related factors in 3T3-L1 pre-adipocytes by the up-regulation of phosphorylated AMPK levels (Ahn et al., 2008).

1.7 Significance and future implications of the study by basic scientists and clinicians

The global outspread of obesity epidemic makes it crucial for Physicians to consider the weight effects of medications being prescribed and to balance the benefits of treatment with the potential for weight gain. Medication-induced weight gain is a significant problem for many treated patients and the weight promoting effects of such medications should be considered in drug choice. The Endocrine Society in 2015 has published clinical practice guidelines for pharmacological management of obesity, including data on medications that cause weight gain. It has further suggested alternatives that are weight-neutral or promote weight loss. Dietary supplements represent an attractive adjuvant to the meditational therapies promoting weight gain because of their low toxicity profiles and accessibility to the general population. The prospect of using natural products to treat obesity and diabetes is still not widely examined. Flavonoids offer a potential alternative treatment strategy for the development of effective and safe anti-obesity and anti-diabetes drugs. Emerging studies have described the promising role of

flavonoids in treating obesity and diabetes as well as their associated metabolic diseases. Positive results from a number of animal and human studies support the use of quercetin in fighting inflammatory disease. This study is a continued evaluation to uncover the exact mechanisms through which quercetin functions in order to satisfactorily address safety concerns. The present research will provide a mechanistic insight into the effects of quercetin as promising and potent antioxidant and anti-inflammatory agent against different molecular mechanisms involved in obesity pathogenesis.

In conclusion, it is also hoped that this study will enhance knowledge and understanding as well as reigniting interest in the anti-inflammatory properties of natural flavonoids and encourage the public to explore vegetarian diets and natural medicines to combat drug-induced obesity.

1.8 Scope of the Study

1.8.1 Working Hypothesis

Commonly used medicines either alone or in combination to treat patients may increase risk of metabolic syndromes.

1.8.2 Main aim

The main aim of this study was to investigate and highlight commonly used medications including rilpivirine and quercetin that might increase the risk of the metabolic syndrome. The study also aimed to determine the modulatory role of quercetin to ameliorate rilpivirine and estradiol induced metabolic alterations.

1.8.3 Specific Aims and Objectives

The specific aims and objectives of this research are as follows:

1. To culture and differentiate human pre-adipocytes into mature adipocytes.
2. To determine the cytotoxicity of various at different time points and dose concentrations.
3. To demonstrate the accumulation of triglycerides during the adipocyte differentiation process by quantitative measurement and staining of the adipose cells.
4. To investigate the levels of inflammatory adipocytokines namely adiponectin, leptin, Interleukin-8 and resistin after adipocyte differentiation.
5. To investigate the levels of oxidative stress markers namely glutathione (GSH), nitric

oxide (NO), superoxide dismutase (SOD) and catalase after adipocyte differentiation.

6. To study the expression of C/EBP- α , PPAR- γ , SREBP-1, FAS, LPL and aP2 genes in the presence or absence of quercetin at mRNA level using RT-PCR.
7. To measure protein levels of adipogenesis markers through quantitative measurement and immunostaining.
8. To undertake a thorough literature search around the subject area, analyze the data and write up the PhD thesis.

1.8.4 Justification of the Study

It is noteworthy that the metabolic syndrome is a cluster of risk factors associated with an increased risk for CVDs and T2DM. The use of certain medications may increase the risk of the metabolic syndrome by either promoting weight gain or altering lipid or glucose metabolism. Health providers should recognize and understand the risk associated with certain medications and appropriately monitor for changes related to the metabolic syndrome. Careful attention to drug choices should be paid in patients who are overweight or have other risk factors for diabetes or CVDs. Several medications like paracetamol, caffeine, rilpivirine, estradiol can induce metabolic alterations by dysregulating adipocyte function, differentiation and disruption of inflammatory as well as oxidative profile at gene and protein levels. Dietary agents such as quercetin when used in supplementation to these drugs can mitigate adipocyte dysfunction by down-regulation of factors involved in the adipogenesis process and modulate the inflammatory and oxidative profile in adipose cells.

As such, this study was designed to specifically investigate the effect of the two metabolic altering drugs, rilpivirine and estradiol, on lipid metabolism in human adipose cells in the presence or absence of quercetin, an anti-inflammatory and anti-oxidant agent. It is justifiable to undertake the study in order to understand any adverse effects of rilpivirine and estradiol during the process of adipogenesis and more so, how quercetin can attenuate these side effects. The results, in turn, may provide knowledge and understanding on drug-induced adipogenesis and also for clinicians to make suitable choices of herbal supplements for therapeutic use.

1.8.5 Approach or scope of the study

This study consists of 7 chapters. Chapter 1 or the introduction chapter is related to the critical and comprehensive review of the subject area including the scope of the study. Chapter 2 describes all the materials employed for the investigation of the study and the methodology and

experimental protocols used in solving the scientific problem. It also contains a short section on statistical data analysis. Chapter 3 is a developmental study relating to the effect of caffeine and paracetamol on adipose cells in the presence or absence of β -naphthoflavone. Chapter 4 investigated the effect of rilpivirine and estradiol on adipogenesis in the presence or absence of β -naphthoflavone. Chapters 5 and 6 investigated the effect of rilpivirine and estradiol, respectively, either alone or in combination with quercetin, a natural flavanoid, at cellular and molecular levels during the process of adipogenesis. Finally, chapter 7 is related to a critical discussion of the findings in chapters 3-6. These different chapters are followed by future studies, limitations, references, appendices with supplementary data and publications.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1 Reagents, cell lines and Consumables

Table 2.1 shows the reagents and consumables used in this study.

Table 2.1: List of reagents, consumables, media and kits employed in this study

Sr.No.	Items	Manufacturers	Catalogue numbers
1.	Adipose Derived Mesenchymal Stem Cells	HiMedia Laboratories	CL007
2.	Adipocyte Differentiation Medium	HiMedia Laboratories	AL521
3.	Mesenchymal Stem Cell Expansion Medium	HiMedia Laboratories	AL512
4.	Human Subcutaneous Pre-adipocytes	Lonza Walkersville, USA	PT-5001
5.	Pre-Adipocyte Basal Medium	Lonza Walkersville, USA	PT-8202
6.	L-Glutamine	Lonza Walkersville, USA	PT-9001H
7.	Gentamicin sulfate amphotericin-B	Lonza Walkersville, USA	PT-4504H
8.	Fetal Bovine serum	Lonza Walkersville, USA	PT-9000H
9.	Insulin	Lonza Walkersville, USA	PT-9003H
10.	Dexamethasone	Lonza Walkersville, USA	PT-9004H
11.	IBMX	Lonza Walkersville, USA	PT-9013H
12.	Indomethacin	Lonza Walkersville, USA	PT-9014H
13.	DMEM, High glucose	HiMedia Laboratories	AL007A
14.	Fetal Bovine serum	HiMedia Laboratories	RM112

15.	Antibiotic-Antimycotic solution	HiMedia Laboratories	A002
16.	Dulbecco's Phosphate Buffered Saline	HiMedia Laboratories	TL1006
17.	Trypsin/EDTA Solution	HiMedia Laboratories	TCL007
18.	Soybean Trypsin Inhibitor Solution	HiMedia Laboratories	TCL068
19.	CytoTox96 Non-Radioactive Cytotoxicity Assay Kit	Promega	G1780
20.	Lipolysis Assay Kit	Abcam	ab185433
21.	Adiponectin Human ELISA Kit	Abcam	ab99968
22.	Human Leptin ELISA Kit	Sigma Aldrich	RAB0333
23.	Human Interleukin-8 ELISA Kit	Sigma Aldrich	RAB0319
24.	Human Resistin ELISA Kit	Sigma Aldrich	RAB0419
25.	DMSO	HiMedia Laboratories	TC185
26.	Rilpivirine	Sigma Aldrich	PZ0180
27.	17 β -Estradiol	Sigma Aldrich	E2257
28.	β -Naphthoflavone	Sigma Aldrich	N3633
29.	Trypsin Protease	Hyclone	SV3003701
30.	AdipoRed Assay Reagent	Lonza Walkersville, USA	PT-7009
31.	Phosphate buffered saline	Hyclone	H30256.01
32.	Human Leptin DuoSet ELISA Kit	R&D Biosystems	DY398
33.	Human IL-8 /CXCL8 DuoSet ELISA Kit	R&D Biosystems	DY208
34.	Human Resistin DuoSet ELISA Kit	R&D Biosystems	DY1359

35.	Human Adiponectin/ACRP30 duoset ELISA kit	R&D Biosystems	DY1065
36.	Dimethyl sulfoxide (DMSO)	Sigma Aldrich	M81802
37.	Rilpivirine	Cayman chemicals	21559
38.	Quercetin	Sigma Aldrich	N3633
39.	Adipogenesis Marker Antibody Sampler Kit	Cell Signalling	12589T
40.	Superoxide Dismutase Assay kit	Cayman Chemicals	706002
41.	Glutathione Assay kit	Sigma Aldrich	CS0260
42.	Nitrate Colorimetric Assay Kit	Cayman Chemicals	780001
43.	Catalase Assay kit	Cayman Chemicals	707002
44.	Alexa Fluor 488 Stain	Thermo Fisher Scientific	A-11034
45.	Hoechst Dapi Stain	Thermo Fisher Scientific	62249
46.	BCA Protein Assay kit	Thermo Fisher Scientific	23225
47.	GenElute Mammalian total RNA MiniPrep kit	Sigma Aldrich	RTN10
48.	cDNA Reverse Transcription kit	Applied Biosystems	4375575
49.	Triglyceride quantification kit	Abcam	Ab65336
50.	Oligonucleotides	AccuvisBio	

Cell lines: 3T3-L1 adipose derived mesenchymal stem cells, Human subcutaneous preadipocytes

Treatment drugs: Paracetamol, caffeine, β naphthoflavone, rilpivirine, estradiol, quercetin.

2.2 Investigational Study Design

In order to confirm the hypothesis proposed in this thesis, human adipose cells were treated with various concentrations of drugs (caffeine, paracetamol, β naphthoflavone, rilpivirine, estradiol, quercetin) either alone or in combination. The study investigated the effect of these drugs at physiological concentrations on adipocyte function, gene expression and inflammation. The study also investigated gene polymorphisms of FABP4 and PPAR variants in diabetic patients in the United Arab Emirates (UAE) population. Investigational methods/techniques such as MTT, LDH, Staining, Inflammatory markers, Oxidative markers, immune-staining, PCR, RFLP, Lipid profiling etc. were employed.

2.3 Biosafety Approval

All ethical and safety consents were obtained by the Ethics Committee of the School of Forensic and Applied Sciences at the University of Central Lancashire, Preston, U.K (Unique reference Number: STEMH 445).

2.4 a. Cell proliferation, seeding and differentiation (Used in Chapters 3 and 4)

In this study, 3T3-L1 adipose derived mesenchymal stem cells (HiMedia) were used to model the individual as well as combinatory effects of rilpivirine and/or 17- β estradiol either with or without BNF in pre-adipocyte differentiation to mature adipocyte cells. The cell line was obtained from HiMedia Laboratories (CL007). These cells were at passage 2 after isolation from human lipo-aspirate collected after liposuction procedure. Upon arrival, morphology and confluency of the cells was checked under the microscope. The cells were then incubated for 3-4 hours to allow floating cells to re-attach to the surface, in a humidified CO₂ incubator with 5% CO₂ and 37 °C temperature. After incubation, the flask was replenished with appropriate amount of complete medium containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter D-glucose, 10% fetal calf serum (FBS) and penicillin /streptomycin (100 units/ml penicillin, 50 μ g /ml streptomycin).

The medium was changed every 2-3 days and the cells were sub-cultured after they reached 70-80% of confluency. For sub-culturing, the spent medium was aspirated gently without disturbing the monolayer and discarded. The cells were then washed with 2-3 ml of Dulbecco's phosphate buffer saline (DPBS) to remove residual medium. 500 μ L of pre- warm trypsin-EDTA

(Ethylenediamine tetra acetic acid) solution was added to detach the adherent cells from the walls of the flask. The flask was incubated for 30 minutes and then observed microscopically to ensure complete detachment of cells. The action of trypsin was neutralized by addition of complete medium and a homogeneous mixture of cells of obtained. The cells were counted using a haemocytometer and an appropriate number was used to seed fresh flasks at recommended seeding density (Approx. 0.125×10^6 cells/cm² of the flask).

After confluence, 3T3-L1 adipocyte differentiation into adipose cells was obtained in the presence of the same culture medium as used before supplemented with adipogenic differentiation medium (HiMedia). This medium contained supplement formulated with induction factors that induced adipogenic differentiation of human mesenchymal stem cells. Table 2.2 illustrated the different media used for maintenance, differentiation and treatment of 3T3-L1 adipocytes in this study.

Table 2.2: Table showing media formulations employed in the study.

Medium	Formulation
Pre-adipocyte Expansion	90% Dulbecco's Modified Eagle's Medium (DMEM), and 10% Bovine Calf Serum
Differentiation	90% DMEM, 10% Fetal Bovine Serum (FBS), 1.0 μ M Dexamethasone, 0.5 mM Isobutyl methyl xanthine (IBMX), and 1.0 μ g/ml Insulin
Adipocyte Maintenance	90% DMEM, 10% FBS, and 1.0 μ g/ml Insulin

For studies on the effects of drugs during adipocyte differentiation, treatment with either 17 β -estradiol, rilpivirine or 17 β -estradiol and rilpivirine in combination and either with or without β -naphthoflavone (BNF) was initiated on day 0 and continued throughout the differentiation period of 10 days. Fresh drugs were added each time the medium was replaced. Stock solutions of 17 β -estradiol and BNF were prepared in DMSO (DiMethyl Sulphoxide) while differentiation medium was used for preparing rilpivirine. They were then aliquoted and stored at -20 °C prior to use.

2.4 b. Cell proliferation and seeding (Used in Chapters 5 and 6)

In the present study, human subcutaneous pre-adipocytes were used to model the effects of rilpivirine and 17 β -estradiol in either the presence or absence of an anti-inflammatory flavanoid, quercetin. The cell line was obtained from Lonza (Cat. # PT5001). These cells were isolated from subcutaneous adipose tissue by enzymatic digestion and selective culturing techniques.

2.4.1 Unpacking and Storage

Upon arrival in the laboratory, the cryovials containing the cells were removed from the dry ice packing and immediately placed in liquid nitrogen. The cells were supplied with a BulletKit™ containing basal medium and a vial of SingleQuots™, which were stored at 4-8°C and -20 °C, respectively. Dulbecco's Phosphate Buffered Saline (DPBS) was stored at ambient temperature. Trypsin/EDTA solution was sterile filtered and stored at -20 °C.

2.4.2 Preparation of Media

Pre-warmed (37 °C), supplemented medium was used for the culturing of pre-adipocytes. Firstly, the external surface of the Pre-adipocyte Basal Medium-2 bottle was decontaminated with 70% v/v of ethanol. The Pre-adipocyte Growth Medium-2 was made by adding the entire contents of the fetal bovine serum (FBS), L-Glutamine, GA-1000 SingleQuots™ to the bottle of Pre-adipocyte Basal Medium-2. The final concentrations of the supplements were 10%, 2 mM, 30 μ g/ml and 1.5 ng/ml, respectively.

2.4.3 Thawing of cells and initiation of culture process

Cryovial containing the cells was removed from liquid nitrogen storage and thawed rapidly in a 37 °C water bath, for no more than 2 minutes. The external surface of the vial was decontaminated with 70% v/v ethanol or isopropanol. Thawed cell suspension was added to 50 ml of pre-warmed Pre-adipocyte Growth Medium-2 and centrifuged at 300 x g for 10 minutes at room temperature. The cells were then washed leaving a minimum of 1 ml of wash at the bottom of the tube. A volume of either 2 or 3 ml of Pre-adipocyte Growth Medium-2 was added to this 1 ml of wash and the pellet was re-suspended. A total of 20 μ l of the cell suspension was finally diluted with an equal amount of 0.4 % trypan blue and a cell count was done to determine % viability.

In order to expand the cells prior to use for assays, the pre-adipocytes were plated with approximately 9,000 cells cm² in 0.2 ml medium cm² growth area in tissue culture flasks. The flasks were placed in CO₂ incubator (Forma™ Series II 3110 Water-Jacketed incubator from Thermo Fisher Scientific) maintained at 37 °C and 5% CO₂. Human primary pre-adipocytes appeared round when first plated and within four hours, greater than 90% of the cells began to flatten and elongate. Within 24-36 hours the cells were adherent, elongated and spindle shaped. As the pre-adipocytes began to divide, they rounded up. The cultures were fed every 3-4 days after plating.

2.4.4 Sub-culturing

The spent media were aseptically removed and discarded from the flasks followed by washing of the attached cell layer with Dulbecco's Phosphate Buffered Saline. The flask was rinsed thoroughly by rocking the flask back and forth several times. The wash solution was then aseptically removed and discarded. Sufficient volume of Trypsin-EDTA solution was then added to cover the cell layer (approx. 25 ml/cm²). Incubation was done at room temperature for 3 minutes and the cells were then observed under the microscope. Once >90 % of the cells were rounded and detached, approximately 0.2 ml/cm² of temperature equilibrated Pre-adipocyte Growth Medium-2 was added to each flask. Pipetting over the cell layer surface several times ensured dispersal of the solution. To remove the trypsin, the cells were centrifuged at approximately 300 x g for 10 minutes at room temperature. The obtained cell pellet was re-suspended in a minimal volume of temperature equilibrated growth medium and the total number of cells was counted using a haematocytometer.

2.4.5 Maintenance and Differentiation

In order to prepare adipocyte differentiation medium, entire contents of the SingleQuots™ containing insulin, dexamethasone, indomethacin and isobutyl-methylxanthine (IBMX) were added to 100 ml of preadipocyte growth medium-2 pre-warmed to 37°C. Pre-adipocytes were induced to differentiate by the addition of 0.1 ml of Adipocyte Differentiation Medium to each well for a period of 8 days.

2.4.6 Adipocyte treatment

In this study, the effect of drugs during adipocyte differentiation, treatment with 17β-estradiol, rilpivirine alone and then in combination with quercetin was initiated on day 0 and continued throughout the differentiation period of 8 days.

2.4.7 Preparation of treatment drugs

Stock solutions of treatment drugs were prepared in DMSO/methanol. They were then aliquoted and stored at -20°C prior to use. Working solutions of the treatment drugs were prepared in pre-adipocyte growth medium-2 by diluting them further as outlined in Table 2.3.

Table 2.3: Table showing the concentrations of treatment drugs used in the study

Treatment Drugs	Stock concentrations	Working concentrations
Rilpivirine	25 mg/ml	0.1 µM, 1 µM, 5 µM, 10 µM, 15 µM and 20 µM
17-β Estradiol	1 mg/ml	0.0001 µM, 0.001 µM, 0.01 µM, 0.1 µM and 1 µM
Quercetin	50 mg/ml	50 µM
Caffeine	1 mg/ml	0.1 mM
Paracetamol	25 µg/ml	0.1 mM
BNF	1 gm/ml	200 µM

2.5 Lactate dehydrogenase Assay for the measurement of cell cytotoxicity

Potential cytotoxic effects of drugs on differentiating human pre-adipocytes was determined after exposure of drugs for 10 days using a CytoTox 96 kit (Promega), which measured the appearance of lactate dehydrogenase (LDH) in the cell culture medium. LDH is a stable cytosolic enzyme normally released upon cell lysis. The released LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay, which resulted in the conversion of a tetrazolium salt (iodonitro-tetrazolium violet; INT) into a red formazan product. The amount of color formed was proportional to the number of lysed cells. Cell viability was assessed on day 0, 5 and 10. Following experimental treatment, supernatant samples were transferred to a 96 well plate and an equal volume of CytoTox 96® Reagent was added to each well and incubated for 30

minutes. Stop Solution was added, and the absorbance signal was measured at 490 nm using a plate reader.

2.6 Adipo Red Staining and Quantitative measurement of triglyceride accumulation

Visualization and quantification of intracellular lipid droplets was done using AdipoRed™ Reagent following the manufacture's protocol. AdipoRed™ Stain was used to determine the effects of test compounds on the differentiation of adipocyte precursors as characterized by the accumulation of intracellular triglycerides.

For the assay, cells were seeded at 10,000 /cm² of the surface area and cultured and differentiated as described above, using appropriate volumes of cell culture media. Immediately prior to the assay, each plate was rinsed with PBS followed by the addition of AdipoRed™, using the volumes as shown in Table 2.4.

Table 2.4: Table showing volumes of PBS and AdipoRed to be used for the assay

	Rinse volume/well	Final volume of PBS/well	Volume of AdipoRed /well
6-well plate	2 ml	5 ml	140 µl
12-well plate	1 ml	2 ml	60 µl
24-well plate	1 ml	1 ml	30 µl
48-well plate	0.4 ml	0.4 ml	12 µl
96-well plate	0.2 ml	0.2 ml	5 µl

The plate was then incubated for 10 to 15 minutes and placed in the fluorimeter. The fluorescence was measured with excitation at 485 nm and emission at 572 nm.

2.7 Quantification of free glycerol

The triglycerides within the cell were lipolysed into glycerol and free fatty acids. Commercially available Abcam's Lipolysis Assay kit (ab185433) was used to measure glycerol released from 3T3-L1 cells colorimetric method. This kit included glycerol assay buffer, glycerol probe, glycerol enzyme mix, glycerol standard, lipolysis assay and wash buffer and was stored at -20°C away from light. The glycerol assay buffer, probe, lipolysis assay and wash buffer were warmed at 37 °C using a water bath prior to use. Glycerol enzyme mix was provided in a lyophilized form and was reconstituted with 20 µL of glycerol assay buffer. Standard curve was prepared by adding 10 µL of 100 mM Glycerol standard to 990 µL of glycerol assay buffer to generate 1mM Glycerol

Standard. Thereafter, volume of 0, 2, 4, 6, 8 and 10 μL of 1 mM glycerol standard was added into series of wells in a 96 well plate to generate 0, 2, 4, 6, 8 and 10 nmol/well of glycerol standards. The volume of each well was adjusted to 50 μL with Glycerol assay buffer. Enough reaction mix consisting of 46 μL of glycerol assay buffer, 2 μL of glycerol enzyme mix and 2 μL of glycerol probe per well was prepared for the number of assays to be performed. A volume of 50 μL of this reaction mixture was added to each well of the 96-well plate containing the standards and the samples. The plate was then incubated at room temperature for 30 minutes protected from light. Absorbance was read at 570 nm in a micro-plate reader.

For calculating glycerol concentrations in the cell samples, zero standard reading was subtracted from all readings and a standard curve was plotted. The corrected sample reading was then applied to the standard curve to get nmol of glycerol amount in the sample wells.

2.8 Quantitative Measurement of Adipokines and Cytokines

Commercially available Human Elisa kits from R&D Systems were used to quantitatively measure the adipocytokines (Adiponectin, leptin, resistin, Interleukin-8) released by adipocytes and accumulated in the cell culture medium at the end of 8 days of differentiation process following the manufacture's protocol. Each of the kit components included human protein standard, human detection antibody, HRP conjugated streptavidin, ELISA colorimetric TMB substrate reagent and Stop solution. The kits were stored at $-20\text{ }^{\circ}\text{C}$ upon receipt. All reagents were brought to room temperature and allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working solutions of Streptavidin-HRP (1:200) and biotinylated mouse anti-human detection antibody were prepared by reconstitution of the supplied vials with Reagent diluent. Capture Antibody was reconstituted with 1.0 ml of PBS. Standards were prepared by reconstitution of each vial with either 0.5 ml of Reagent Diluent or 1X PBS. A seven-point standard curve using 2-fold serial dilutions in Reagent Diluent was prepared as described by the Manufacturer.

Plate Preparation: In order to prepare the micro-plate for assay, capture antibody was diluted to the working concentration in PBS without carrier protein and a volume of 100 μ l was immediately coated onto each well. The plate was then sealed and incubated overnight at room temperature. Each well was aspirated and washed with a 400 μ l volume of wash buffer using an auto washer (HydroFlex™ micro-plate washer from Tecan). This process was repeated two times for a total of three washes. Complete removal of liquid at each step was ensured each time for good performance. After the last wash, any remaining Wash buffer was removed by aspirating or by inverting the plate and blotting it against clean paper towels. Wells were blocked by the addition of 300 μ l reagent diluent to each well followed by incubation at room temperature for a minimum of 1 hour. Washing step was repeated as earlier and the plates were made ready for sample addition.

Assay Procedure: A volume of 100 μ l of either sample or standards in the reagent diluent, in duplicates, were added per well and incubated for 2 hrs at room temperature. Aspiration and washing steps were repeated as described earlier followed by addition of 100 μ l of the Detection Antibody, diluted in Reagent Diluent, to each well. The plate was then covered with an adhesive strip and incubated 2 hours at room temperature. After this, a volume of 100 μ l of the working dilution (1:200) of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature. Thereafter, aspirate / wash steps were repeated. A volume of 100 μ l of substrate solution was added to each well and the plate was incubated for 20 minutes at room temperature and any exposure to direct light was avoided. A volume of 50 μ l of stop solution was added to each well and the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined immediately, using a micro plate reader (Molecular Devices from Life technologies™ equipped with Softmax pro7 Version 4.3 software) set to 450 nm.

Calculation of results: For obtaining the results, average zero standard optical density was subtracted from mean absorbance of each set of duplicate standards, controls and samples. Standard curve was prepared by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve through the points on the graph. Sample concentrations were calculated using the equation of the standard curve.

2.9 Quantitative measurement of oxidative stress markers

Markers of oxidative stress (Superoxide dismutase, Glutathione, Nitric oxide and Catalase) were quantified using the cell lysates from the cultures using commercially available kits.

2.9.1 Superoxide Dismutase (SOD) levels

SOD levels were determined using the Superoxide Dismutase Assay kit (Cat. # 706002) purchased from Cayman chemicals, following the manufacturer's protocol. The kit contained Assay buffer (10X), Sample buffer (10X), Radical detector, 1 vial of SOD Standard and Xanthine oxidase. Assay and Sample buffers were diluted to 1X using HPLC-grade distilled water. A volume of 50 μ l of radical detector (tetrazolium salt solution) was diluted with 19.95 of diluted Assay buffer and covered with foil. To prepare the SOD standard, vial containing 100 μ l of bovine erythrocyte SOD (Cu/Zn) was thawed on ice. A volume of 20 μ l of this solution was diluted with 19.8 ml of 1X Sample buffer to obtain the SOD stock solution and standards were prepared serially in glass tubes ranging from A-G as outlined in table 2.5.

Table 2.5: Preparation of Superoxide Dismutase Standards

Tubes	SOD Stock (μ l)	Sample buffer (μ l)	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

In order to perform the assay, SOD standard wells were prepared by adding 200 μ l of the diluted radical detector and 10 μ l of Standard (tubes A-G) per well. Sample wells were prepared by

adding 200 µl of the diluted Radical Detector and 10 µl of sample to the wells. Reaction was initiated by adding 20 µl of diluted Xanthine Oxidase to all the wells. The plate was then kept on a shaker for 30 minutes at room temperature and absorbance was read at 440-460 nm using a plate reader. Average absorbance for each standard and sample was calculated by subtracting the sample background absorbance from the sample. Linearized rate was obtained by dividing standard A's absorbance by itself and then with absorbance values of all other standards and samples. This linearized SOD standard rate was plotted as a function of final SOD Activity. SOD activity was calculated by using the equation obtained from the linear regression of the standard curve and substituting the linearized rate for each sample (Figure 2.1).

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}} \right) \times \frac{0.23 \text{ ml}}{0.01 \text{ ml}} \right] \times \text{sample dilution}$$

Figure 2.1 : Equation for the calculation of SOD activity.

2.9.2 Glutathione Assay

This assay was performed using the Glutathione Assay Kit from Sigma-Aldrich (Cat. #CS0260) following the manufacture's protocol. For the assay, stock solution of 5, 5'-Dithiobis (2-nitrobenzoic acid) [DTNB] was prepared by dissolving 8 mg of DTNB with 5.33 ml of DMSO to make a 1.5 mg/ml solution. NADPH stock solution was prepared by dissolving 25 mg of NADPH in 0.625 ml of water to give a 40 mg/ml solution. An amount of 2.5 gm of powdered 5-sulfosalicylic acid (SSA) was completely dissolved in 50 ml of water to prepare a 5% stock solution. Glutathione (GSH) Standard Stock solution (10 mM) was prepared by dissolving the entire contents of glutathione reduced standard vial in 0.1 ml of water. For the working solutions, Assay buffer was diluted 5-fold to make 1X concentration. Glutathione Reductase Enzyme Solution was prepared by diluting it to a final volume of 250 µl with 1X assay buffer. 10 µl of NADPH stock solution was added to 2.5 ml of 1 X Assay buffer to make its working solution. To make the working mixture 8ml of 1X Assay buffer, 228 µl of diluted enzyme solution and 228 µl of DTNB Stock solution were mixed together. Glutathione standard solutions were prepared by diluting an aliquot of the Glutathione (GSH) Standard stock solution by 200-fold with 5% SSA

solution. Standards were prepared by adding 50 μM of glutathione solution in the first well and then subsequently diluting it serially as mentioned in table 2.6.

Table 2.6: Table showing the preparation of glutathione standard solutions by serial dilution of the 50 μM glutathione solution

Well Numbers	1	2	3	4	5
GSH concentration (μM)	50	25	12.5	6.25	3.125
GSH solution (μl)	50	25(from well 1)	25(from well 2)	25(from well 3)	25(from well 4)
5% SSA (μl)	None	25	25	25	25
Nmoles GSH in a 10 μl sample	0.5	0.25	0.125	0.0625	0.0312

In order to prepare the samples for the assay, cell extracts were washed with PBS and at least 1×10^8 cells were suspended per ml of PBS and transferred to a micro centrifuge tube. The cells were then centrifuged at 600 x g and supernatant was removed. The volume of the pellet was measured and 3 volumes of 5% SSA solution was added and vortexed. The suspension was then freeze-thawed twice using liquid nitrogen and a 37° C bath. It was then left for 5 minutes at 2–8 °C. The extract was then centrifuged at 10,000 x g for 10 minutes. Volume of supernatant was measured, and the sample was kept at 2–8 °C.

For the assay procedure, plate reader was set to 412 nm with kinetic read at 1-minute intervals for 5 minutes. The reaction scheme was set up according to table 2.7. Each test was performed in duplicate.

The first 2 wells contained only 10 μl of the 5 % 5-sulfosalicylic acid solution as a reagent blank. A total of 10 μl glutathione standard solutions and unknown samples in duplicate were added into separate wells of the plate. A volume of 150 μl of working mixture was added to each well using a multichannel pipette followed by 5 minutes of incubation at room temperature. After

incubation, 50 µl of diluted NADPH Solution was added to each well. Plate reader was used to measure the absorbance in each well.

Table 2.7: Table showing Reaction Scheme for the GSH assay

	Mix and Incubate 5 minutes			Start
Sample measured	Sample Volume	5% SSA	Working Mixture	NADPH (0.16 mg/ml)
Reagent Blank	-	10 µl	150 µl	50 µl
Standard Curve (various dilutions)	10 µl	-	150 µl	50 µl
Unknown sample	x µl	10-x	150 µl	50 µl

To compute the results, values from the glutathione standard solutions were used to determine the standard curve and calculate ΔA_{412} /min equivalent to 1 mole of reduced glutathione per well.

Concentrations of nano-moles of GSH in the unknown samples were calculated using the following formula (Figure 2.2):

$$\text{nmoles GSH per ml of sample} = \frac{\Delta A_{412}/\text{min}(\text{sample}) \times \text{dil}}{\Delta A_{412}/\text{min}(1 \text{ nmole}) \times \text{vol}}$$

Figure 2.2: Equation for the calculation of GSH activity.

where:-

ΔA_{412} /min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction).

ΔA_{412} /min (1 nmole) = slope calculated from the standard curve for 1 nmole of GSH

Dilution = dilution factor of original sample

Volume = volume of the reaction in ml

2.9.3 Nitric Oxide Assay

Cayman's Nitrate/Nitrite colorimetric Assay kit (Cat. #780001) was used for the measurement of total nitric oxide following the manufacturer's protocol. The kit contained Nitrate/Nitrite Assay Buffer, Nitrate Reductase Enzyme Preparation, Nitrate Reductase Cofactor Preparation, Nitrate Standard, Griess Reagent R1, Griess Reagent R2 and 96 well micro-plate with cover sheet.

The kit was stored at -20°C upon receipt. Before the Assay procedure, working reagents were prepared by either dilution or reconstitution with a suitable reagent. Nitrate/Nitrite assay buffer was prepared by diluting the contents of the supplied vial to 100 ml of ultrapure water. Contents of nitrate reductase enzyme preparation and nitrate reductase cofactors Preparation were reconstituted with 1.2 ml of Assay buffer and kept on ice during use. Freezing and thawing of these solutions were limited to one time only. Vial containing lyophilized powder of nitrate standard was reconstituted with 1.0 ml of assay buffer and stored at 4°C. Griess Reagents R1 and R2 were ready to use.

Preparation of Nitrate Standard Curve: Nitrate standard curve was prepared in order to quantify samples of nitric oxide concentrations. Stock standard (200 μ M) was prepared by adding a volume of 0.9 ml of assay buffer to 0.1 ml of reconstituted nitrate standard. The standard curve for nitrate was prepared by addition of reagents to the plate wells as shown in Table 2.8.

Table 2.8: Table showing preparation of nitrate standard concentrations

Wells	Nitrate Standards (μ l)	Assay Buffer (μ l)	Final Nitrate Concentrations *(μ M)
A1, A2	0	80	0
B1, B2	5	75	5
C1, C2	10	70	10
D1, D2	15	65	15
E1, E2	20	60	20
F1, F2	25	55	25
G1, G2	30	50	30
H1, H2	35	45	35

* The concentration was calculated for the final 200 μ l of assay volume after addition of the Griess Reagents.

Assay Procedure: A volume of 200 μ l of water or assay buffer (to be used as blank) and 80 μ l of sample was added to the wells. After this, a total of 10 μ l of the Enzyme Cofactor Mixture followed by 10 μ l of the nitrate reductase mixture was added to each of the wells (standards and unknowns). The plate was covered and incubated for 2 hours at room temperature. After the incubation time, a volume of 50 μ l of Griess Reagent R1 followed by 50 μ l of Griess Reagent R2 was added to each of the wells. The plate was left for 10 minutes at room temperature for the development of colour. Absorbance was read at 540 nm using a plate reader.

Calculations: Mean absorbance values of the blank wells were subtracted from the absorbance values of all the other wells. Corrected absorbance values at 540 nm were plotted as a function of nitric oxide concentration. Sample nitric oxide concentrations were determined using the

following formula (Figure 2.3):-

$$[\text{Nitrate} + \text{Nitrite}] (\mu\text{M}) = \left(\frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left(\frac{200 \mu\text{l}}{\text{volume of sample used } (\mu\text{l})} \right) \times \text{dilution}$$

Figure 2.3: Equation for the calculation of nitric oxide concentration.

2.9.4 Catalase Activity

The enzymatic activity of catalase was determined following Cayman's Catalase Kit (Cat. # 707002) using the cell lysate. The kit was stored at 4°C upon receipt. Before the assay, stock solutions of catalase assay buffer and catalase sample buffer were diluted to a 1 X concentration using HPLC grade water. Vial containing lyophilized powder of catalase positive control was reconstituted using 2 ml of diluted Sample buffer. Working solution of catalase hydrogen peroxide was prepared by diluting 40 µl of this solution with 9.96 ml of HPLC grade water. To prepare the samples, cells were collected by centrifugation at 1000-2000 g for 10 minutes at 4 °C. The resulting cell pellet was homogenized on ice in 1-2 ml of cold buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA) followed by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was removed and stored on ice for the assay. For the preparation of formaldehyde standards, a volume of 10 µl of Catalase Formaldehyde Standard was diluted with 9.99 ml of diluted sample buffer to obtain a 4.25 mM formaldehyde stock solution which was further serially diluted as described in table 2.9.

For performing the assay, formaldehyde standard wells were prepared by adding a volume of 100 µl of diluted assay buffer of standard (tubes A-G) per well in the designated wells on the plate. Positive control wells were prepared by adding 100 µl of diluted Assay buffer, 30 µl of methanol, and 20 µl of diluted Catalase (Control) to two wells. Sample wells were prepared by addition of 100 µl of diluted assay buffer, 30 µl of methanol, and 20 µl of sample to duplicate wells. Reaction was initiated by adding 20 µl of diluted hydrogen peroxide to all the wells. The plate was covered and incubated on a shaker for 20 minutes at room temperature. The reaction was terminated by adding 30 µl of potassium hydroxide followed by addition of 30 µl of catalase purpald (Chromogen) to each well. Incubation was done for 10 minutes at room temperature on the shaker. After this, a volume of 10 µl of catalase potassium periodate was added to each well

and incubation was done for 5 minutes at room temperature on a shaker. Finally, the absorbance was read at 540 nm using a plate reader. Rate of the reaction was determined by subtracting the average absorbance of each standard and sample from the average absorbance of standard A. The corrected absorbance of standards was plotted as a function of final formaldehyde concentration (μM). Formaldehyde concentration of the samples was calculated using the equation obtained from the linear regression of the standard curve after substituting corrected absorbance values for each sample (Figure 2.4).

Table 2.9: Table showing the preparation of formaldehyde standard solutions by serial dilution.

Tubes	Formaldehyde (μl)	Sample Buffer (μl)	Final Concentrations (μM formaldehyde)
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

$$\text{Formaldehyde } (\mu\text{M}) = \left[\frac{\text{Sample absorbance} - (\text{y-intercept})}{\text{Slope}} \right] \times \frac{0.17 \text{ ml}}{0.02 \text{ ml}}$$

Figure 2.4: Equation for the calculation of formaldehyde.

Catalase Activity of the samples was calculated using the following formula (Figure 2.5):

$$\text{CAT Activity} = \frac{\mu\text{M of Sample}}{20 \text{ min.}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

Figure 2.5: Equation for the calculation of catalase activity.

2.10 Immuno-fluorescence staining

For immuno-fluorescence staining, cells were grown, treated, fixed and stained directly in 96 well black-coloured plates with clear bottom. Following differentiation with treatment drugs, the media were aspirated, and the cells were fixed by covering them to a depth of 2-3 mm with 4% formaldehyde diluted in 1X PBS for 15 minutes at room temperature. After fixation, the fixative was aspirated, and the cells were rinsed three times in 1X PBS for 5 minutes each. Cells were then blocked by incubating them in blocking buffer containing 5% normal goat serum and 0.3% triton X-100 in 1X PBS for around 1 hour. Primary antibodies were prepared fresh in antibody dilution buffer containing 1% BSA and 0.3% triton-X in 1X PBS according to the dilutions mentioned in table 2.10. A volume of 50 µl of primary antibody was added to each well. The microplate was left to incubate overnight at 4°C. Washing step was repeated as earlier and 50 µl of goat anti-rabbit fluorochrome-conjugated secondary antibody, Alexa Fluor 488 from Thermo Fisher Scientific (Cat. # A-11034), diluted to a concentration of 1:1000 in Antibody dilution buffer was added for 1-2 hour at room temperature in the dark. Wells were then carefully washed with 1X PBS to remove unbound probes and counter stained with a sufficient volume of Hoechst 33342 (Thermo scientific Cat. # 62249) working solution to completely cover the samples. An aluminum foil was placed over the micro plate to protect it from light and

incubated at room temperature for 5-10 minutes. Fluorescence images were acquired using an Olympus IX53 inverted microscope equipped with a CCD camera (Olympus, Tokyo, Japan) and computer assisted image analysis using CellSens imaging software.

Table 2.10: Table showing primary antibody dilutions of different adipogenic markers used for immune-staining

Primary Antibodies	Dilutions
Perilipin	1:200
Cebp α	1:100
Fabp4	1:200
Ppar	1:100
Fas	1:50
Acetyl CoA carboxylase	1:200

2.11 Protein Extraction and Quantification

2.11.1 Extraction of total Protein

Human subcutaneous adipose cells were cultured in tissue culture dishes as described previously in sections 2.4.4 and 2.4.5. At approximately 80-90% of confluency, the cells were treated with different concentrations of drugs used in the study and differentiated into mature adipocytes for a period of 8 days. At the end of the treatment period the media were removed from the dishes, and the cells washed twice with PBS in order to ensure that all non-cell protein was removed from the plates. The cells were then transferred to 15 ml tubes and centrifuged at 15,000 rpm for 10 minutes. The supernatant was removed and 150 μ l of radio-immuno-precipitation assay (RIPA) buffer was added to each tube in order to lyse the cells. The samples were then incubated on ice for 30 minutes and then centrifuged again for another 10 minutes at 15,000 rpm and 4°C temperatures. Finally, the supernatant containing the proteins was transferred to new eppendorf tubes and stored at -20 °C.

2.11.2 Quantification of total protein

Total proteins in the samples were quantified using BCA (Bicinchoninic Acid) Protein Assay kit (Cat. # 23225) taken from Thermo Scientific following the Manufacturer's protocol. A set of diluted standards were prepared using 1 ml ampoule of 2 mg/ml of provided Bovine Serum Albumin (BSA) as shown in table 2.11.

Table 2.11: Table showing preparation of BSA standards for protein quantification

S.No	BSA conc. (μ g/ml)	BSA Volume (μ l)	Diluent (1X PBS) volume (μ l)	Total Volume (μ l)
1.	2000	190	0	190
2.	1500	52.5 of (1)	17.4	70
3.	1000	80 of (1)	80	160
4.	500	80 of (3)	80	160
5.	250	80 of (4)	80	160
6.	125	80 of (5)	80	160
7.	25	20 of (6)	80	100
8.	0	0	80	80

BCA Working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. For the assay, a volume of 25 μ l of replicate standards and the samples were pipetted into micro plate wells. This was followed by the addition of 200 μ l of the prepared

working reagent to each well. The plate was mixed thoroughly using a plate shaker, covered and incubated at 37°C for 30 minutes. It was then cooled to room temperature and absorbance was measured at 562 nm on a plate reader.

2.12 Gene Expression Analysis

2.12.1 Total RNA Isolation

Approximately 2×10^6 adipose cells were cultured in T-75 tissue culture flasks in replicates as described previously. At approximately 80-90 % Of confluency, the cells were treated with different concentrations of drugs used in the study and differentiated into mature adipocytes for a period of 8 days. RNA was isolated using GenElute Mammalian Total RNA Miniprep kit from Sigma-Aldrich (Cat. #RTN10) following the manufacturer's instructions. Attached adipose cells were trypsinised from the culture flasks and centrifuged to form a cell pellet. This was followed by lysis of the cells by adding a volume of 500 µl of Lysis solution/ 2-mercaptoethanol to each of the culture vessels. The cell lysate was then filtered to remove the cellular debris. A filtration column was assembled with a 2 ml collection tube and the lysed cells from the previous step were carefully pipetted onto the filtration column. Centrifugation was done at 15,000 rpm for 2 minutes and the filtration column was discarded. An equal volume (500 µl) of 70 % ethanol was added to the filtered lysate and vortex-mixed thoroughly. A binding column was assembled with a 2 ml collection tube and 700 µl of the lysate/ethanol mixture was pipetted onto the binding column. Centrifugation was done at maximum speed of 14,000 rpm. Flow-through liquid was discarded while the collection tube was retained. A volume of 500 µl of the wash solution was added into the column and centrifuged at maximum speed for 15 seconds. The binding column was then transferred to a fresh 2 ml collection tube and a volume of 50 µl of the elution buffer was pipetted into the column followed by centrifugation at maximum speed for 15 seconds. Elution step was repeated with a second 50 µl volume of the elution buffer, collecting both elutes in the same tube. It was then stored at -80 °C until quantification.

2.12.2 RNA Quantification

Thermo Scientific™ NanoDrop 2000 was used to quantify and assess purity of RNA. Micro-volume sample size (0.5-2.0 µL) was pipetted directly onto an optical measurement surface and RNA was quantified. Once the measurement was complete, the surface was simply wiped with a lint-free lab wipe. All samples were measured in duplicates.

2.12.3 cDNA conversion and Reverse transcriptase PCR

Semi-quantitative RT-PCR method was used to assess gene expression. The cDNAs were synthesized using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems as per the protocol provided by manufacturer. The kit contained 10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100 mM), MultiScribe™ Reverse Transcriptase and RNase Inhibitor. The kit was stored at -20°C upon receipt and thawed on ice before use. To synthesize single-stranded cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems), 2X Reverse Transcription Master Mix was added to 2 µg of the extracted RNA for each sample. Thereafter, 2X RT master mix (per 20 µL reaction) was prepared by using the kit components before preparing the reaction plate according to table 2.12.

Table 2.12: Table showing reaction volumes of kit components for the preparation of 2X RT Master mix

Components	Volume/Reaction(µl)
10x RT Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H ₂ O	3.2
Total volume	10

In order to prepare the cDNA RT reactions 10 µL of 2x RT master mix was pipetted into each 200 µL PCR tube followed by 10 µL of RNA sample. The tubes were then vortex-mixed using mini-spin to ensure thorough mixing. Before loading into the thermal cycler, the tubes were briefly centrifuged to spin down the contents and to eliminate any air bubbles. In order to perform reverse transcription, thermal cycler was programmed according to the conditions in table 2.13.

Table 2.13: Thermal cycling conditions for RT-PCR

	Step 1	Step2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

The reaction volume was set to 20 µL and the reverse transcription run was started. The product purity was confirmed by a dissociation curve analysis. The mRNA levels of the target genes were normalized to the values of β -actin, and presented as qualitative changes relative to controls. Table 2.14 showed the list of primer sequences for adipogenic genes used in the study.

Table 2.14: Table showing a list of primer sequences for adipogenic genes used in the study

Genes	Primer sequences	
	Forward	Reverse
aP2	5'-TCA CCT GGA AGA CAG CTC CT-3'	5'-AAT CCC CAT TTA CGC TGA TG-3'
C/EBP α	5'-GAA CAG CAA CGA GTA CCG GGT-3'	5'-GCC ATG GCC TTG ACC AAG GAG-3'
FAS	5'-GCT TTG CTG CCG TGT CCT TCT-3'	5'-TCT AGC CCT CCC GTA CAC TCA-3'
LPL	5'-AGT AGA CTG GTT GTA TCG GG-3'	5'-AGC GTC ATC AGG AGA AAG G-3'
PPAR γ	5'-CCA GAG CAT GGT GCC TTC GCT-3'	5'-CAG CAA CCA TTG GGT CAG CTC-3'
SREBP-1c	5'-CGC TAC CGG TCT TGT ATC ATT G-3'	5'-TTG CTT TTG TGT GCA CTT CG-3'
β -Actin	5'-AGG CTG TGC TGT CCC TGT AT-3'	5'-ACC CAA GAA GGA AGG CTG CA-3'

2.13 Triglyceride Quantification

Measured using Triglyceride Quantification Assay Kit from Abcam (ab65336) following the manufacturer's protocol. The kit was stored immediately at -20 °C in dark upon receipt. The kit components included triglyceride assay buffer, triglyceride probe, lipase, triglyceride enzyme mix and 1 mM of triglyceride standard. Both triglyceride enzyme-mix and lipase were reconstituted in 220 µl volume of triglyceride assay buffer and kept on ice during the assay. A

0.2 mM triglyceride standard was prepared by diluting 100 μ L of the 1 mM standard in 400 μ L of assay buffer. Using this 0.02 mM standard, standard curve dilutions were prepared as described in table 2.16 below.

Table 2.15: Serial dilution for preparation of standards for triglyceride assay

Standard #	TG Standard (μ L)	Assay buffer (μ L)	Final Volume Standard in well (μ L)	End amount of TG Standard in well (nmol/well)
1	0	150	50	0
2	30	120	50	2
3	60	90	50	4
4	90	60	50	6
5	120	30	50	8
6	150	0	50	10

For the assay procedure, all materials and prepared reagents were gently agitated and equilibrated to room temperature prior to use. All standards, controls and samples were assayed in duplicate. For setting up the reaction wells, a total volume of 50 μ L of standard dilutions and samples were used followed by addition of 2 μ L of lipase. The wells were mixed thoroughly and incubated for 20 minutes at room temperature to convert triglyceride to glycerol and fatty acid. A total of 50 μ L of triglyceride reaction mix (46 μ L of triglyceride assay buffer + 2 μ L of triglyceride probe + 2 μ L of triglyceride enzyme mix) was added to each of the standard and sample wells. A master mix of the reaction mix was prepared to ensure consistency. Wells were mixed and incubated at room temperature for 60 minutes protected from light. Readings were taken on a microplate reader at an absorbance of OD 570 nm. For data analysis, corrected absorbances were calculated by subtracting mean value of blank (standard # 1) from all standards and samples. Corrected values for each standard were plotted as a function of the final concentration of triglyceride. Corrected sample OD was applied to the

standard curve to get triglyceride (B) amount in the sample wells. Concentration of triglyceride in nmol/ μ L (mM) in the test samples was calculated using the following equation:

$$\text{Triglyceride concentration} = B/V * D$$

Where:

B= amount of triglyceride in the sample well calculated from standard curve in nmol.

V= sample volume added in the sample wells (μ L)

D= sample dilution factor

2.14 Statistical analysis of data

Data were analyzed statistically using SPSS Version 25 software. The means of the data were presented either with the standard error mean (SEM) or the standard deviation (SD) where possible. The results were analyzed using one-way ANOVA to determine the significance of the mean between the groups. Values of $p < 0.05$ were considered significant.

Chapter 3: (*A developmental Study*)

**Effects of Caffeine, Paracetamol and β -Naphthoflavone on the
Lipid Content and Differentiation of Human Adipose Derived
Mesenchymal Stem Cells *In Vitro*.**

3.1 Introduction

Obesity is a disorder in which excessive accumulation of lipids in adipose tissue is characteristic. White Adipose Tissue (WAT) contains adipocyte precursors which under adipogenic conditions undergo adipogenesis to become mature adipocytes. Subsequent excessive proliferation of these adipocytes and their enlargement- hyperplasia and hypertrophy respectively, are characteristic features of obesity. Adipose tissue not only acts as fat storage, but also as an endocrinal organ and it secretes various important adipocytokines such as adiponectin (Desai et al., 2013; Meena et al., 2014). Obesity is also characterized by chronic low-grade inflammation due to over nutrition that stresses and overloads signaling networks as well as inflammatory cytokines which are released by WAT. In turn, this affects immune and metabolic systems leading to inflammation (Iyer et al., 2010).

It has also been linked with higher risks of cardiovascular diseases and muscular-skeletal disorders and its rise is now witnessed globally. Approximately, 65% of the world's population now resides in geographic areas where being overweight and obese causes more fatalities than being underweight. It has been suggested that individuals that suffer from obesity (BMI > 30) may be more prone to develop metabolic abnormalities. Due to the widespread effect of obesity, the search for compounds that can effectively be employed in the production of anti-obesity drugs and treatments is now becoming more desirable.

A lot of research has been conducted to study the effects of different chemicals and substances on adipogenesis, lipolysis and their subsequent effects on obesity and fat accumulation. One of the widely studied drugs is caffeine, being the most used pharmacologically active agent (Tofovic et al., 2002). Caffeine is available in several over the counter cosmetics and a large variety of foods and drinks that are consumed by people every day contains caffeine. This makes the prospect of finding its therapeutic effects more desirable. When used in combination with other drugs (such as ephedrine) or other herbal supplements and concoctions, it has been shown to help in the treatment of obesity and promotion of weight loss. This is due to the metabolic effects that are exhibited by caffeine (Bracale et al., 2014). Caffeine is a compound that acts as an antagonistic inhibitor of adenosine in the sympathetic nervous system. However, caffeine actually causes an increase in cell activity as opposed to adenosine binding which slows down its regulation (Bracale et al., 2014). Due to this, it prevents the breakdown of cyclic adenosine monophosphate (c-AMP). Higher concentrations of c-AMP causes an increase in protein kinase A (PKA) that subsequently increases the production of ATPase – an enzyme that is

involved in energy synthesis- and this, in turn, causes an overall increase in the body's metabolic activity (Sassone-corsi, 2012).

A key element of the adipogenic pathway related to c-AMP is the c-AMP-response element binding protein (CREB), whereby its activation has been shown to be necessary for the up-regulation of several other adipogenic regulators. These include CCAAT/ enhancer-binding protein (C/EBP) whose isoform C/EBP β is an initiator of adipogenesis through the expression of C/EBP α and more importantly peroxisome proliferator activated receptor γ (PPAR γ) transcription factors which work together to allow adipocyte differentiation (Fox et al., 2006; Darlington et al., 1999).

PPAR γ is involved heavily in lipid metabolism and is expressed in both white and brown adipose tissue. The deletion of PPAR γ in mice sustained with a high fat diet revealed significant differences in the pattern and amount of lipid accumulation. They exhibited a loss of white adipose tissue mass, lower overall body fat percentage and an increase in adiponectin levels (Jones et al., 2005). This shows the importance of PPAR γ in the adipogenic pathway and its effect on expression of adiponectin which is inversely proportional to fat mass and plays a role in inflammation and energy homeostasis.

Adiponectin is an adipocytokine that causes an increase in fatty acid oxidation (Yamauchi et al., 2002). Transgenic murine models designed to over-express adiponectin in adipose tissues were found to have a decrease in both adipose mass and adipocyte size due to a higher level of energy expenditure and glucose utilization caused by higher levels of expressed adiponectin (Bauche et al., 2007). The relationship between adiponectin levels and lipid accumulation has also been demonstrated in various murine obesity models as well as human obesity studies (Piñeiro et al., 2005; Bauche et al., 2007).

These findings possibly provide a therapeutic possibility to battle obesity through the use of adiponectin enhancing agents – such as caffeine. Studies on mice models that were placed on a high fat diet and then treated with caffeine have demonstrated an increase in lipolysis of fat droplets (Han et al., 1999). Other studies have linked higher adiponectin serum levels and decreased lipoprotein cholesterol with increased intake of caffeine rich products such as coffee (Yamashita et al., 2012). The metabolic pathways and mechanics of caffeine in the human body are also important to be considered when attempting to understand its effects.

In addition to the use of caffeine to sustain high c-AMP levels, the use of paracetamol (acetaminophen) due to its cyclooxygenase inhibiting mechanism can also affect adipocyte

differentiation by effectively attenuating the expression and activity of PPAR γ . The mechanism of action for paracetamol is most likely to occur through the inhibition of prostaglandin (PG) synthesis via the inhibition of cyclooxygenase-2 enzymes (COX-2) (Anderson, 2008). The role of PGs and subsequent hormones and enzymes synthesized is essential in adipocyte differentiation. PGs are lipid mediators that undergo synthesis through the action of different enzymes. Arachidonic acid is first released by phospholipases (PLA₂s) and undergoes conversion to PGH₂ by one of the two COX isozymes (COX-1 or COX-2). The PGH₂ that is produced is metabolized into different PGs that are involved in adipocyte differentiation which causes the activation of PPAR γ (Ghoshal et al., 2011; Ueno et al., 2011). Therefore, bearing this expression mechanism in mind- the inhibition of COX-2 would prevent or decrease the conversion of arachidonic acid to PGH₂ which will have the ultimate effect of reducing and/or inhibiting adipocyte differentiation. COX-2 activation has also been found to be significantly involved in the development of an inflammatory response in adipose tissue (Hsieh et al., 2010) and has been linked to contribute in brown adipocyte differentiation in mice (Ghoshal et al., 2011).

Despite this proposed mechanism, no conclusive research has yet provided a definitive answer to the role of COX-2 inhibition or over-expression in adipocyte expression. Studies conducted on 3T3-L1 cell lines showed that different adipocyte specific markers which included PPAR γ and adiponectin were found to be decreased after the use of COX inhibitors (Mazid et al., 2006). COX-2 deficient murine models which were supplied with a high fat diet showed lower body mass than their wild type counterparts along with higher metabolic activity, lower PPAR γ , lower inflammatory markers and significantly higher levels of adiponectin (Ghoshal et al., 2011). These variables coincide with other results linking them to increased lipolysis and lower body fat and basal metabolic index (BMI), thus suggesting that the use of a COX-2 inhibitor (such as paracetamol) can possibly provide a protective treatment opportunity against obesity and can be employed as a future anti-obesity medication. However, a minor percentage of opposing research showed an activation of PPAR γ in adipocyte cell lines when treated with non-steroidal anti-inflammatory (NSAID) drugs such as indomethacin (Lehmann et al., 1997).

Aryl Hydrocarbon Receptor (ahR) is a ligand activated transcription molecule that has been shown to regulate adipogenic factors and is involved in the induction of detoxifying enzymes such as cytochrome P450 (Yoshinari et al., 2006). Synthetic flavonoid α -Naphthoflavone is classified as an ahR antagonist and has been shown to promote adipogenesis and lipid accumulation in 3T3-L1 cells in vitro (Wang et al., 2015). Inversely, utilizing ahR agonists such as

β -Naphthoflavone (BNF) are hypothesized to have an opposite effect and effectively inhibit or minimize lipid accumulation. BNF has been shown to be an effective inducer of important detoxifying enzymes such as CYP1A1 through the translocation of ahR receptor bound to BNF (Yoshinari et al., 2006).

Aims: Taking into consideration the variation of information in the literature about caffeine and paracetamol about obesity, this developmental chapter of the study was designed to investigate the effects of caffeine and paracetamol either alone or combination in the absence and presence of the β -naphthoflavone, an ahR antagonist, on the differentiation of human adipose derived mesenchymal stem cells, a model of obesity.

3.2 Materials and Methods

As described in Chapter 2.

3.3 Results

3.1 Morphological Examination

Upon arrival, the cells appeared healthy and had a stretched out, irregular shape which is characteristic of pre-adipocytes (Figure 3.1A). After 48 hours of addition of differentiation medium, the cells began to take on a more regular spherical appearance and clumps had begun to form indicating the initiation of the differentiation process (Figure 3.1B). Following the addition of differentiation media 2 after a period of 48 hours, the cells began to differentiate further to form mature adipocytes (Figure 3.1C). The formation of fat droplets in the cells and increased cell clumping was observed indicating successful differentiation.

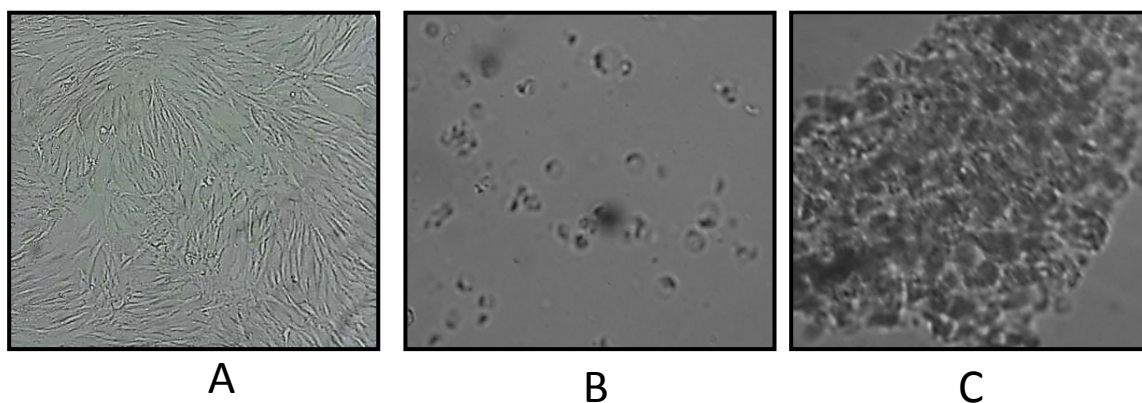


Figure 3.1: Figure showing the progression of the differentiation process in adipose cells from immature preadipocytes to mature adipose cells. Photographs are typical of 20 or more images.

A. Undifferentiated cells.

B. Adipose cells after the addition of differentiation medium 1 over a period of 48 hours.

C. Adipose cells after the addition of differentiation medium 2 over a period of 48 hours.

3.2 Measurement of cell viability by MTT Assay

MTT assay was used to determine cell viability (cell death or proliferation) after the addition of drugs. Results are typical of 6 such different experiments.

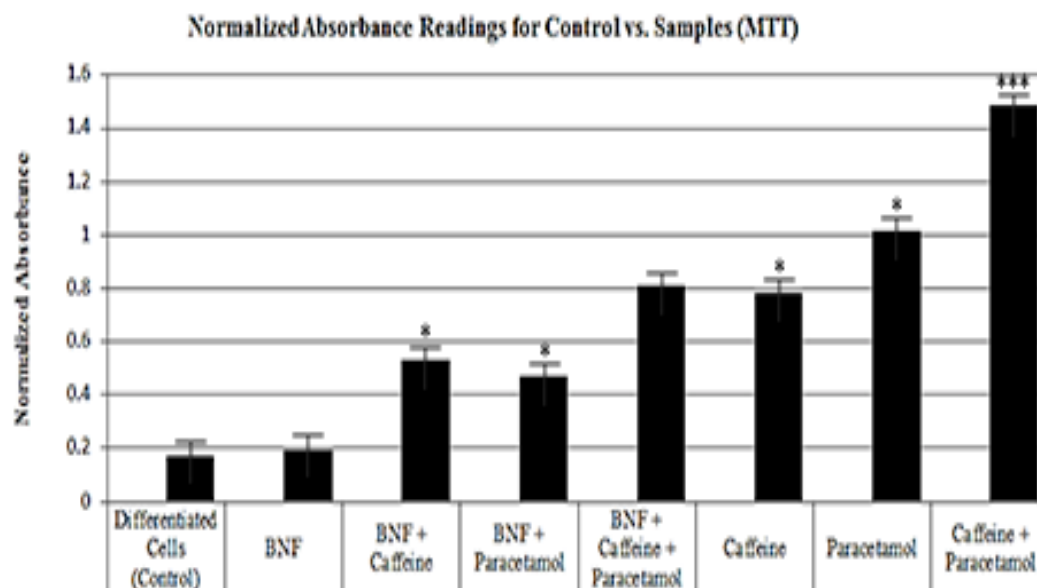


Figure 3.2: MTT assay absorbance readings of all wells compared to the control.

Figure 3.2 illustrates the results of this test compared to the control. As observed, the wells treated with drugs had overall higher absorbance readings than the control indicating that their viability was higher. All samples show readings higher than the control. The last four bars in particular – BNF + caffeine + paracetamol, caffeine, paracetamol and caffeine + paracetamol – show the most significant ($p < 0.05$) difference when compared to the control. This observation indicates that all the cells were viable and that drug interactions may have contributed to this enhanced viability or cell proliferation. Data are mean \pm SD, $n=5$, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ were considered significant for test as compared to control.

3.3 Quantitative measurement of Triglyceride Concentrations

Adipose cells were treated with individual as well as in combination with either caffeine, paracetamol and BNF. Supernatant was collected on the 10th day of culture and triglycerides were measured using commercially available kits.

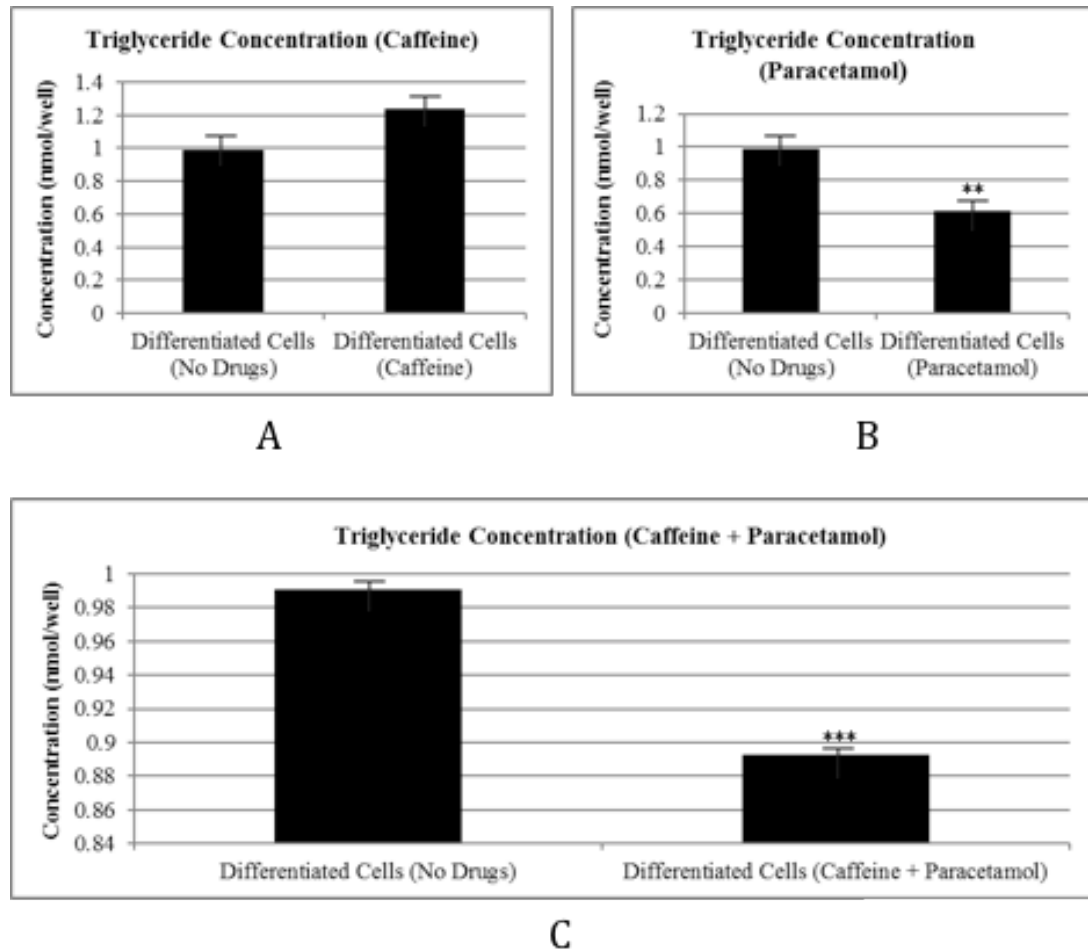


Figure 3.3: Bar graphs showing comparative effects of caffeine and paracetamol independently and in combination on the triglyceride concentration after differentiation of adipose cells for a period of 10 days.

A. Elevated triglyceride concentration was seen when cells were treated with caffeine independently. This was the only case of elevation seen across all samples.

B. Reduced triglyceride levels when cells are exposed to paracetamol.

C. The effect of combining both caffeine and paracetamol appears to null the different effects exhibited by each drug independently giving lower triglyceride content than the control.

Data are mean \pm SD, n=5, *p<0.05 was considered significant for test as compared to control.

The cells that were treated only with caffeine had higher triglyceride concentration as compared to the control (Figure 3.3 A) as well as in comparison to all other independent and combinatory drug treatments. In contrast, significantly notable differences in triglyceride concentrations were observed in the well treated with paracetamol (Figure 3.3 B) whereby it had lower triglyceride content than the control. Interestingly, when both paracetamol and caffeine were used together, their individual effects seemingly balanced out and an overall lower concentration of triglyceride was expressed as compared to independent caffeine treatment (Figure 3.3 C).

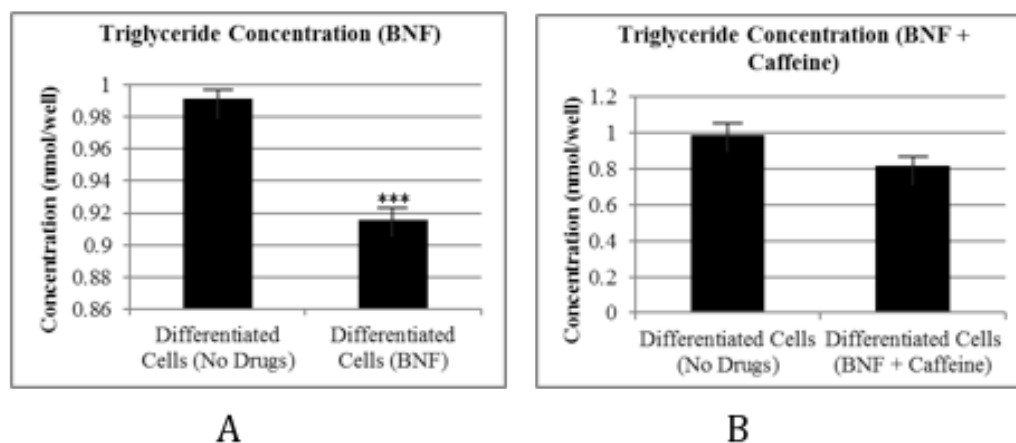


Figure 3.4: Bar graphs showing comparative effects of BNF alone and in combination with caffeine on the triglyceride concentration after differentiation of adipose cells for a period of 10 days.

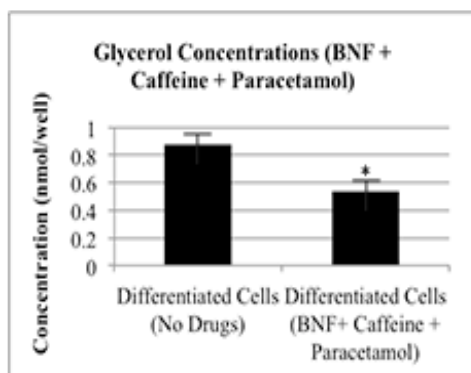
A. Cells treated with BNF independently showed a significantly lower concentration of triglycerides than the control with no drugs.

B. When cells are treated with both BNF and caffeine, the triglyceride concentration is raised when compared with the use of BNF alone. This provides further indication that caffeine causes an increase in triglyceride content as it seemingly attenuated the suppressive effect of BNF on triglyceride concentration of cells. Data are mean \pm SD, $n = 5$, $*p < 0.05$ for test compared to control.

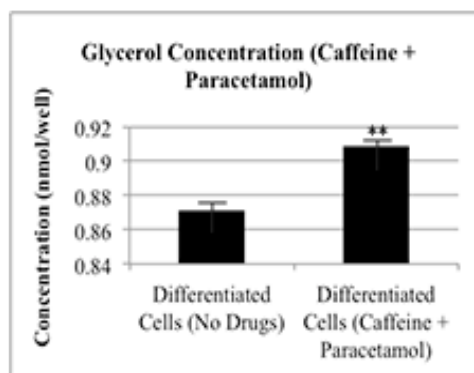
The treatment of cells using BNF independently displayed a dramatically lower triglyceride content compared to the control (Figure 3.4A) as well as in comparison to the cells treated with BNF + caffeine (Figure 3.4B).

3.4 Quantitative measurement of Free Glycerol Concentrations

(A)



(B)



(C)

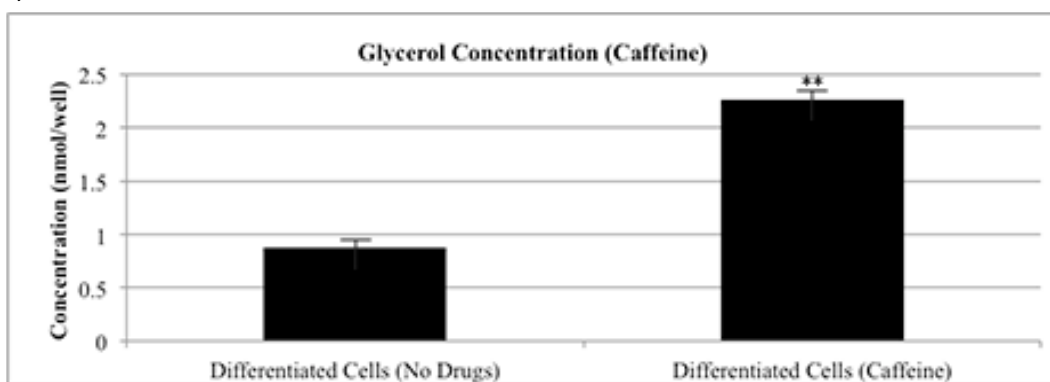


Figure 3.5: (Images A-C): Bar graphs showing the concentrations of glycerol with different combinations of treatment drugs. Data are mean \pm SD; n=5; * <0.05 while ** $p<0.01$.

A. Illustrates the glycerol concentration when BNF, caffeine and paracetamol are used in combination. The levels of glycerol are lower than the control under these conditions and therefore can suggest an inhibited or minimized rate of lipolysis.

B. Glycerol concentration is significantly raised when BNF is excluded and only caffeine and Paracetamol are used in conjunction with each other. The glycerol content is higher than the preceding condition (5A) and the control.

C. An approximate 2.5 times higher glycerol concentration observed in the well treated with caffeine alone when compared to the control. The use of caffeine independently showed the highest glycerol concentration overall indicating higher lipolysis levels. Data are mean \pm SD, n=5, * $p<0.05$ for test compared to control.

Free glycerol content was used as an indicator of lipolytic activity within the cells as it is released when triglycerides are hydrolyzed and broken down. Bearing the above in mind, the wells treated with paracetamol, BNF and BNF + Caffeine had almost equal glycerol levels as compared to control. Alternatively, the cells treated with BNF + caffeine + paracetamol (Figures 3.5 A), had significantly lower glycerol concentrations than the control. Cells treated with caffeine + paracetamol showed higher glycerol levels (Figure 3.5 B). Most notably, out of all the samples taken, the well containing caffeine had the highest glycerol concentration overall with it being approximately 2.5 times more glycerol than the differentiated control (Figure 3.5 C).

3.5 Discussion

Triglycerides are biological molecules composed of a glycerol backbone and fatty acids which are stored in adipocytes (Prattes et al., 2000). Their content within cells can be used as a direct indicator of adipogenesis, that is, the higher the content, the more adipogenesis and adipocyte differentiation are occurring. The hydrolysis of triglycerides leads to the process of lipolysis- thereby releasing glycerol (Ahmadian et al., 2007). Therefore, glycerol concentrations can be used as direct indicators of lipolytic activity in cells. The present results demonstrate that caffeine, paracetamol, BNF and their varying combinations may all have effects on the processes of adipogenesis and lipolysis.

The triglyceride and glycerol concentrations of cells treated solely with caffeine reflect lipolytic properties. The use of caffeine resulted in the highest glycerol concentration among all tested drugs and combinations- the concentration was approximately 2.5 times higher than the differentiated control cells but with no addition of drug. As expected, triglyceride contents of caffeine were lower than glycerol- however; the triglyceride content of cells with caffeine was higher than all other samples. These traits could be an indicator that caffeine contains potent lipolytic effects, but no significant effect on the process of adipogenesis itself. Perhaps, caffeine can have an effect on existing triglycerides but cannot inhibit their formation. Conversely, paracetamol appears to have no significant effect on the process of lipolysis, but it can reduce triglyceride concentrations indicating that the independent use of paracetamol may contain anti-adipogenic effects and thereby preventing triglyceride formation. The suggested lipolytic and anti-adipogenic effects of caffeine and paracetamol are further emphasized when examining their combined effect on pre-adipocytes differentiation. The triglyceride and glycerol

concentrations under this condition are very similar to one another indicating a sort of homeostasis where the enhanced lipolytic effects expressed by caffeine are compensated by increased adipogenesis directed by paracetamol.

The use of BNF to treat cells has varying effects based on whether it is used either alone or in combination with other drugs. When BNF is used independently, triglyceride concentrations are reduced in comparison with differentiated control cells. However, glycerol concentrations under treatment with BNF are equal to the differentiated control. To further examine the consequences of utilizing BNF, it was used as an additional supplement along with caffeine, paracetamol and their combination. When BNF is used with caffeine, the pronounced lipolytic effects of caffeine appear to become diminished and glycerol concentration levels become lower than the differentiated control. This effect on glycerol is also seen when BNF is used to supplement paracetamol and the combination of caffeine and paracetamol. This may suggest that BNF has inhibitory effects on lipolysis and it can attenuate any potent lipolytic effects expressed by the use of caffeine and paracetamol. In contrast to the observed inhibitory effect of BNF on lipolysis, its effects on adipogenesis favour anti-adipogenic properties. When used to treat cells independently, the triglyceride concentration of cells is lower than the differentiated control. This observation implied anti-adipogenic effect is also observed when BNF is used to supplement caffeine and the combination of caffeine and paracetamol. The triglyceride concentrations under these two conditions are reduced as compared to those expressed in the same conditions without BNF supplementation. This observation provides further indication that BNF can be used to significantly enhance anti adipogenic properties of caffeine and paracetamol through the attenuation of triglyceride expression. MTT analysis showed increased cell viability when treated with drugs in comparison to the differentiated control lacking drugs. This suggests that the use of drugs was not harmful or toxic to cells and may have had a role to play in enhancing their viability.

In conclusion, the results provide preliminary evidence towards the anti adipogenic and lipolytic properties of caffeine, paracetamol and BNF. The presence of underlying mechanisms that can potentially express these properties merits further research and exploration. BNF is known to induce detoxifying CYP450 enzymes which may be a contributing factor to the anti adipogenic effects. Future research to study the gene expression of CYP450 could provide further evidence of the adipogenic process that occurs during the differentiation of pre-adipocytes under the presence of different drugs.

Chapter 4

Effects of Rilpivirine, 17 β -Estradiol and β -Naphthoflavone on the inflammatory status of release of adipocytokines in 3T3-L1 adipocytes *in vitro*.

4.1 Introduction

Obesity is a major global health problem and if left untreated, it may lead to major complications (Zimmet et al., 2005). It is attributed to a number of factors including over-eating, sedentary lifestyle, lack of physical activity, genetics, diets rich in carbohydrate, frequency of eating, hormonal and various psychological factors as well as others. Some medications are also associated with weight gain. These include certain anti-depressants, anti-convulsants, some diabetes medication and certain hormones including leptin, insulin, estrogens, androgens and growth hormones (Ness-Abramof et al., 2005; Ratliff et al., 2010).

Rilpivirine is a prescription medicine for the treatment of HIV infection in adults and children. Although the drug was approved in 2011 by the U.S., the Food and Drug Administration (FDA), its approval document included fat re-distribution among its warnings and precautions. These contradictory results show its effect on the adipose tissue distribution and function which have been presented by previously conducted clinical trials (Cohen et al., 2011; Molina et al., 2011; Wilkin et al., 2012; Tebas et al., 2014). On the other hand, 17 β -estradiol is a major component of hormonal contraceptives and it acts primarily as an agonist of the estrogen receptor (ER). Several studies have provided evidence of a hormonal regulation of pre-adipocyte proliferation (Hausman et al., 2001; Thorn et al., 2007). Estrogen promotes subcutaneous fat and regulates adipose tissue distribution through its receptors, ER α and ER β (Matelski et al., 1985; Kuiper et al., 1996). Moreover, 17 β -estradiol has been shown to have stimulatory effects on proliferation of female rat pre-adipocytes (Dieudonne et al., 2000) and in human pre-adipocytes (Anderson et al., 2001). The British HIV guideline for the management of HIV infection indicated that rilpivirine is an appealing option to consider in women of childbearing potential. At the same time, it also warns of the significant pharmacokinetic and pharmacodynamic interactions that have been reported between the anti-retroviral (ARV) drugs and hormonal agents. Inducers of hepatic enzymes by ARVs may result in increased breakdown of estrogen that can compromise contraceptive efficacy (Taylor et al., 2012). In addition, both of these drugs are known to alter lipid metabolism and fat distribution in the body (Rimsky et al., 2013). Therefore, a mechanistic insight into the elucidative role of concomitant use of rilpivirine and estradiol would provide more robust and reliable prediction of their potential interactions on the adipogenesis pathway.

Organic pollutants such as β -naphthoflavone (BNF) tend to escape from the body metabolic system and accumulate in the white adipose tissue (WAT), thereby affecting its physiological role, and thus leading to the development of obesity-related disorders. The mechanisms

through which such chemicals modulate the differentiation, metabolism, and secretory function of adipocytes are numerous (Müllerová et al., 2007; Elobeid et al., 2008; Uemura et al., 2008; Feige et al., 2007). Aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor that participates in a variety of metabolic processes, including detoxification and adipogenesis, has been reported to be functionally present in rodent adipose tissue (Yoshinari et al., 2004, 2006). Activated AhR modulates expressions of various genes involved in xenobiotic-metabolizing pathways, such as cytochrome P4501A1 (CYP1A1) (Shimada et al., 1998; Arinç et al., 2000; Safe et al., 2013). CYP1A induction may be used as a biomarker of exposure to toxic substances in order to ascertain whether in vitro models could be useful in investigating the bio activation potential of environmental contaminants.

4.2 Aims: Therefore, this study was designed to investigate the effect of rilpivirine and estradiol either alone or in combination on the adipose cells measuring free glycerol and a number of inflammatory markers including leptin, and adiponectin, resistin and IL-8 which are associated with adipocyte function. In another series of experiments, rilpivirine and estradiol were combined with an environmental pollutant, BNF to determine any inhibitory or stimulating effects.

4.3 Materials and Methods

As discussed in Chapter 2.

4.4 Results

Figure 4.1 shows human pre-adipocytes that were differentiated in culture in the presence of different concentrations of rilpivirine or estradiol either alone or combined for a period of 10 days. The results show that untreated cells on day 0 display spindle shaped, fibroblast-like appearance with undetectable lipids. However, with the progression of adipogenesis, mature adipocytes attain a round shape and become laden with lipids.

4.4.1 Morphological examination

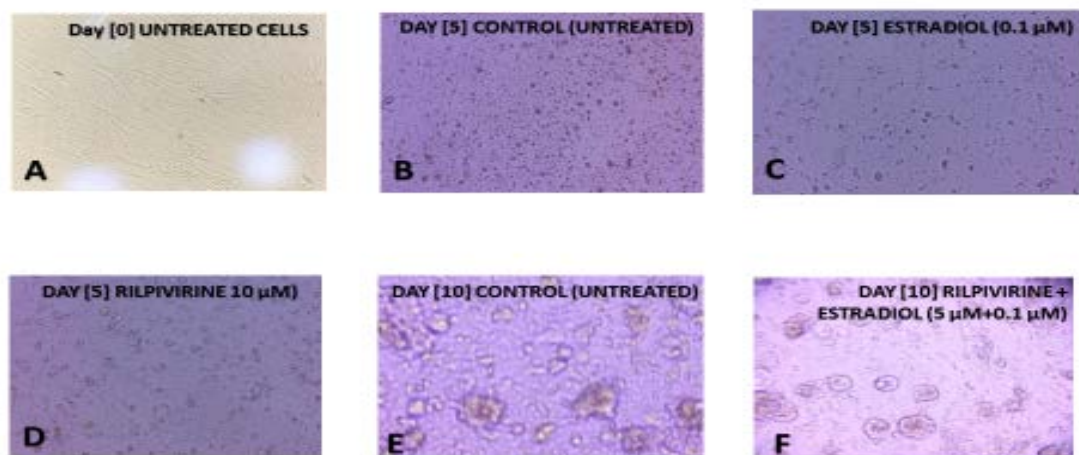


Figure 4.1: Confocal image photographs (A-F) taken at 10X magnification of human adipocytes showing the effects of rilpivirine, estradiol and their combination on the morphology of the cells. Each image is typical of 6 such different experiments.

4.4.2 Cell viability assessment

Cell viability was assessed for the two series of 3 different experiments measured in duplicates (n=6) by employing lactate dehydrogenase assay. The results are shown in tables 4.1 and 4.2. Values were expressed as the percentage test cells surviving compared to control cells. The results in these tables show no significant ($p>0.05$) difference in cell viability with drug treatments compared untreated control.

Table 4.1: Cell viability measurement of human pre-adipocytes treated with rilpivirine and estradiol either alone or during combination. Pre-adipocytes were treated with the different concentrations of drugs for 10 days. Data are expressed as % viability \pm SD relative to the control, $p < 0.05$ for treatment compared to control; $n = 6$.

		Day 0	Day 5	Day 10
Drugs	Doses (μ M)	% Viability \pm SD		
Control	0	97.23 \pm 0.003	97.14 \pm 0.002	96.47 \pm 0.006
Rilpivirine	1	97.27 \pm 0.006	96.92 \pm 0.003	96.71 \pm 0.006
Rilpivirine	5	96.89 \pm 0.013	95.65 \pm 0.05	95.17 \pm 0.01
Rilpivirine	10	97.23 \pm 0.006	96.25 \pm 0.01	95.82 \pm 0.01
Estradiol	0.01	97.27 \pm 0.006	96.05 \pm 0.02	95.68 \pm 0.01
Estradiol	0.05	97.13 \pm 0.002	96.56 \pm 0.03	95.86 \pm 0.01
Estradiol	0.1	97.23 \pm 0.003	95.04 \pm 0.003	95.68 \pm 0.008
Rilpivirine+Estradiol	1+0.01	97.23 \pm 0.005	95.69 \pm 0.04	95.47 \pm 0.001
Rilpivirine+Estradiol	5+0.05	97.58 \pm 0.013	96.84 \pm 0.004	94.71 \pm 0.01
Rilpivirine+Estradiol	10+0.1	97.23 \pm 0.003	95.89 \pm 0.04	94.75 \pm 0.01

Table 4.2: Cell viability of human pre-adipocytes treated with rilpivirine and estradiol either alone or in combination but in the continuous presence of 200 μ M BNF. Pre-adipocytes were treated with the different concentrations of drugs for 10 days. Data are expressed as % viability \pm SD relative to the control; note that $p > 0.05$ for control compared to treatments; $n = 6$.

		Day 0	Day 5	Day 10
Drugs	Dosage (μ M)	% mean viability \pm Standard Deviation (SD)		
Control	0	97.23 \pm 0.004	96.61 \pm 0.075	96.64 \pm 0.03
Rilpivirine + BNF	1 + 200	97.06 \pm 0.005	96.86 \pm 0.04	96.22 \pm 0.004
Rilpivirine +BNF	5 + 200	97.23 \pm 0.003	96.84 \pm 0.01	96.82 \pm 0.01
Rilpivirine + BNF	10 + 200	97.23 \pm 0.003	96.72 \pm 0.007	96.22 \pm 0.001
Estradiol + BNF	0.01+ 200	97.16 \pm 0.009	96.74 \pm 0.01	96.47 \pm 0.004
Estradiol+ BNF	0.05+ 200	97.09 \pm 0.004	95.26 \pm 0.04	96.54 \pm 0.01
Estradiol +BNF	0.1+ 200	97.23 \pm 0.006	95.26 \pm 0.05	96.82 \pm 0.01
Rilpivirine+Estradiol+BNF	1+0.01+ 200	97.09 \pm 0.003	94.66 \pm 0.01	96.43 \pm 0.007
Rilpivirine+Estradiol+BNF	5+0.05+ 200	96.96 \pm 0.002	94.78 \pm 0.05	96.78 \pm 0.02
Rilpivirine+Estradiol+BNF	10+0.1+ 200	97.09 \pm 0.004	96.44 \pm 0.01	96.43 \pm 0.01

4.4.3 Measurement of levels of free glycerol and adipocytokines in untreated and treated adipocytes after 10 days

Human adipose cells were treated with various concentrations of rilpivirine and estradiol either alone and in combination and supernatant was collected after 10 days of differentiation. Quantitative measurement of free fatty acids and adipocytokines was done using commercially available kits.

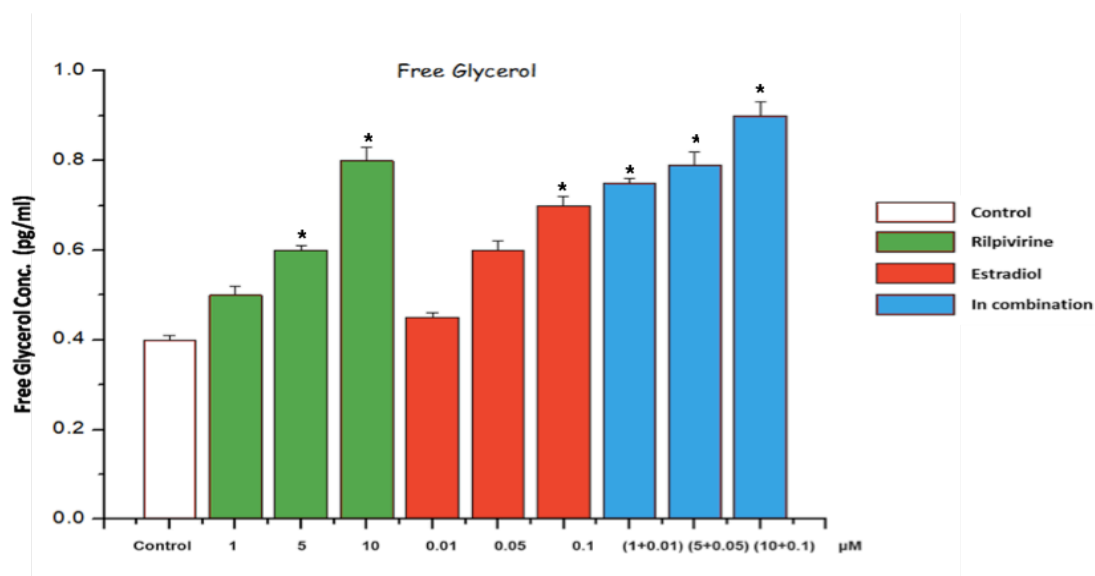


Figure 4.2: Bar charts showing the effects of different concentrations of either rilpivirine or estradiol alone or in combination on the release of free glycerol from human adipocytes following 10 days in culture. In this and subsequent figures, values are presented as means \pm SD from duplicate set of experiments expressed relative to values from untreated control cells. $n=6$; * $p<0.05$ for control compared to drug treatments.

The data presented in figure 4.2 show the effects of different concentrations of either rilpivirine or estradiol alone or in combination on the release of free glycerol from human adipocytes. The results show that all drug treatments can evoke significant ($p<0.05$) increases in free glycerol release from human adipocytes compared to untreated control, except for 0.01 μ M and 0.005 μ M estradiol, which induces only a small increase in free glycerol.

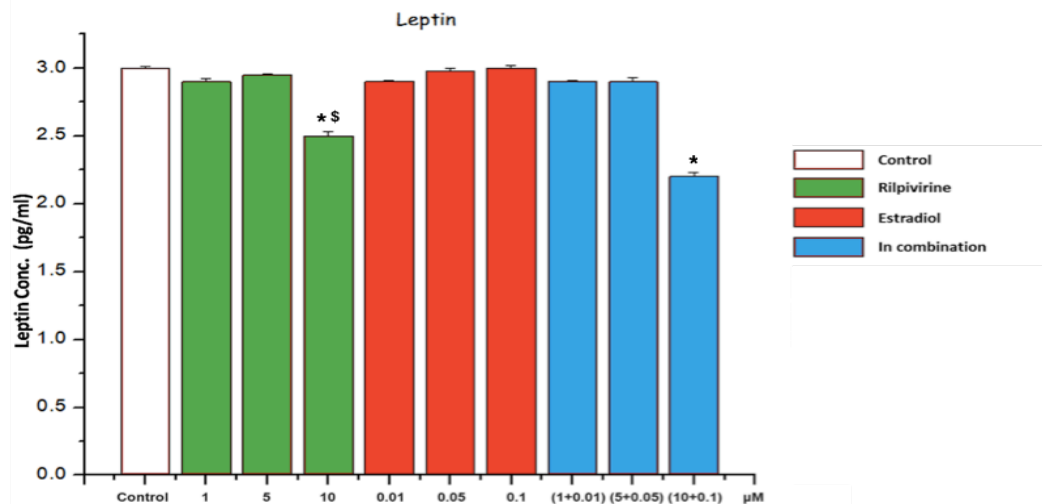


Figure 4.3: Bar charts showing the effect of rilpivirine and estradiol either alone or in combination on the release of leptin by human adipocytes in culture for 10 days. Values are means \pm SD; * $p < 0.05$ compared to untreated control cells; $n = 6$.

Figure 4.3 shows the effect of rilpivirine and estradiol either alone or in combination on the release of leptin by human adipocytes in culture for 10 days. The results show the different types of drug treatments had little or no effect on leptin release, except for 10 μ M rilpivirine alone or when it was combined with 0.1 μ M estradiol when a significant ($p < 0.05$) decrease in leptin release were observed.

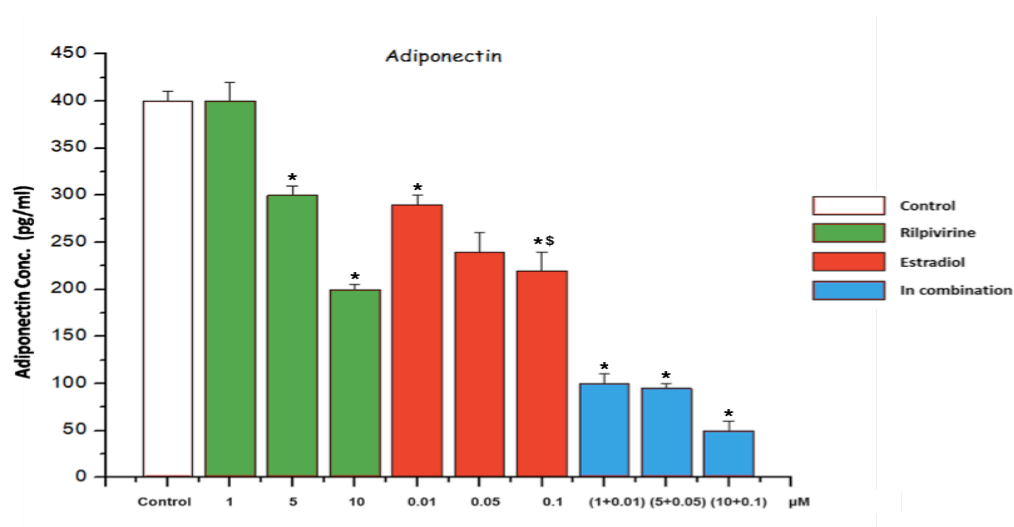


Figure 4.4: Bar charts showing the effect of rilpivirine and estradiol either alone or in combination on the release of adiponectin from human adipocytes in culture for 10 days. Data

are means \pm SD=6; * $p<0.05$ for all concentrations of the drugs compared to control, except for 1 μ M rilpivirine.

The results presented in figure 4.4 show the effect of rilpivirine and estradiol either alone or in combination on the release of adiponectin from human adipocytes in culture for 10 days. The results show significant ($p<0.05$) decreases in adiponectin release for all drug treatments compared to untreated control, except for 1 μ M rilpivirine and 0.05 μ M estradiol.

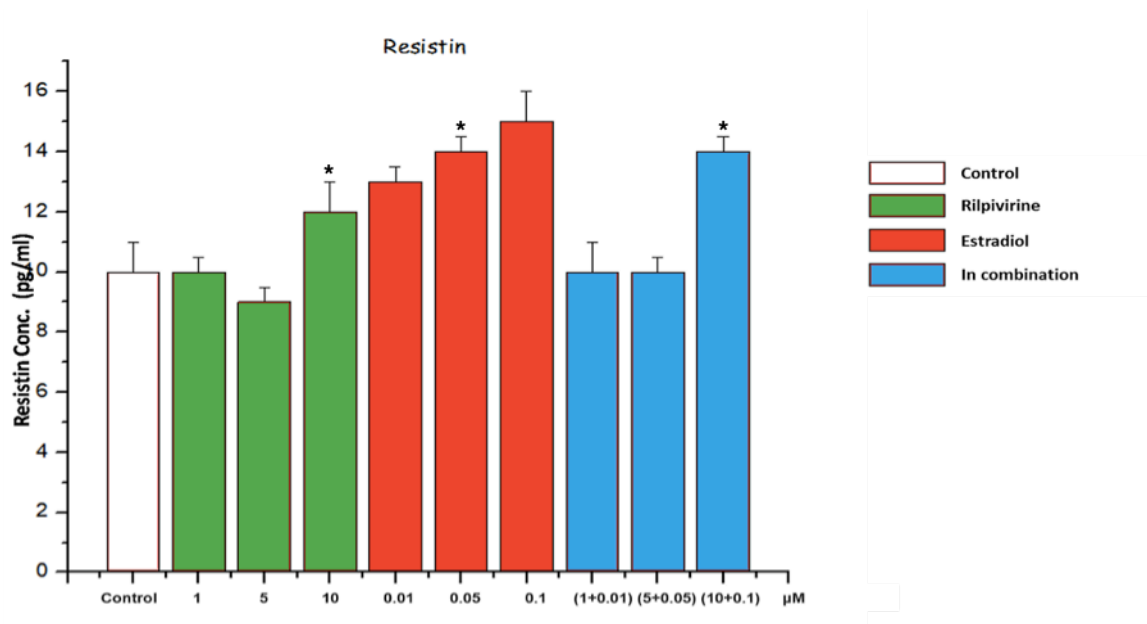


Figure 4.5: Bar charts showing the effect of rilpivirine and estradiol either alone or in combination on the release of resistin from human adipocytes in culture for 10 days. Values are means \pm SD; $n=6$; * $p<0.05$ for all drug treatments compared to untreated control except for 1 μ M and 5 μ M rilpivirine alone and when rilpivirine (1 μ M and 5 μ M) was combined with estradiol (0.1 μ M and 0.5 μ M).

The results show effect of rilpivirine and estradiol either alone or in combination on the release of resistin from human adipocytes in culture for 10 days. The data reveal significant ($p<0.05$) release in resistin for all drug treatments compared to untreated control except for 1 μ M and 5 μ M rilpivirine and rilpivirine (1 μ M and 5 μ M) combined with estradiol (0.1 μ M and 0.5 μ M).

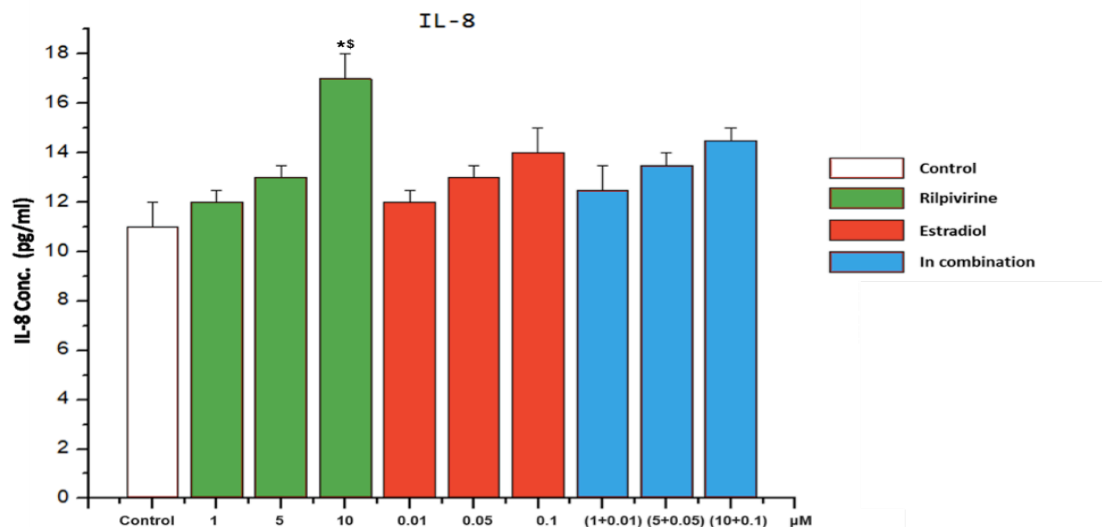


Figure 4.6: Bar charts showing the effect of rilpivirine and estradiol either alone or in combination on the release of interleukin-8 from human adipocytes in culture for 10 days. Data as means \pm SD; n=6; *p<0.05 for 10 μ M rilpivirine compared to untreated control.

Figure 4.6 shows the effect of rilpivirine and estradiol either alone or in combination on the release of IL-8 from human adipocytes in culture for 10 days. The data reveal a significant (p<0.05) release in IL-8 for only 10 μ M rilpivirine compared to untreated control. The other drug treatments only evoked small increases (but not significant, p>0.05) in IL-8 compared to untreated control cells.

4.4.4 Measurement of free glycerol and adipocytokines from human adipocytes treated with various concentrations of rilpivirine and estradiol either alone or in combination but in the continuous presence of 200 μ M β -naphthoflavone (BNF)

In this series of experiments, the human adipocytes were pretreated with BNF followed by treatment with either rilpivirine or estradiol alone or during their combination employing different doses. The rationale was to ascertain whether BNF might exert an attenuating or potentiating effect on lipolysis and the release of adipocytokines from human adipocytes.

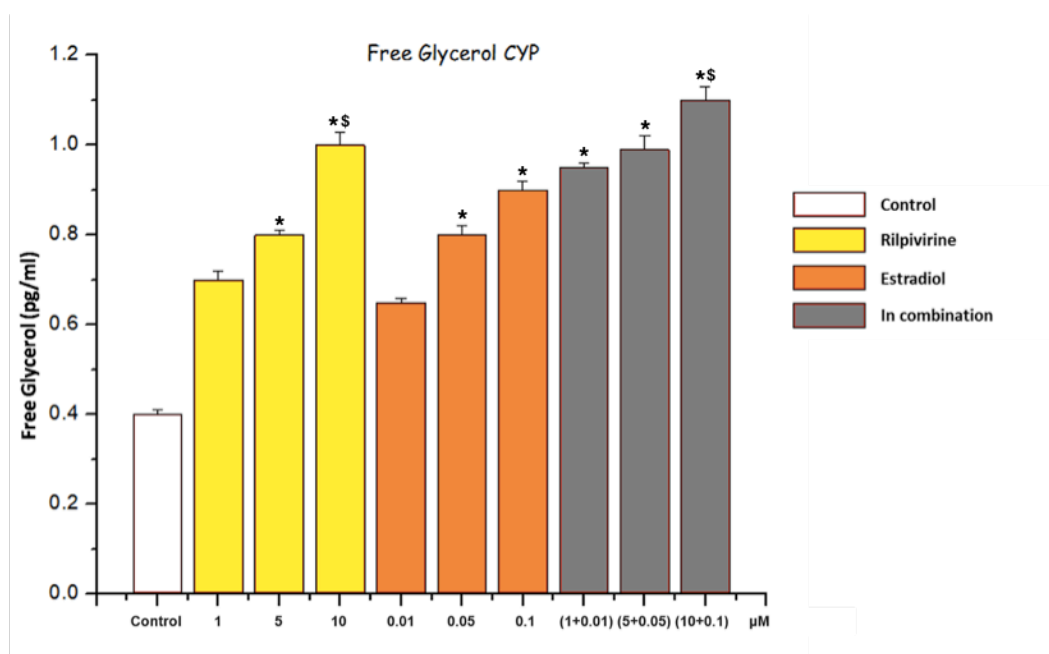


Figure 4.7: Bar charts showing the effect of different concentrations of rilpivirine and estradiol either alone or following combination in the presence of 200 μ M of BNF on the release of free glycerol from human adipocytes in culture over 10 days with the drugs. In this and subsequent figures human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Values are means \pm SD; n=6; *p<0.05 for all drug treatments compared to untreated control cells.

Figure 4.7 shows the effect of different doses of rilpivirine and estradiol either alone or following combination in the presence of 200 μ M of BNF on the release of free glycerol from human adipocytes in culture over 10 days with the drugs. The results show that all drug treatments can evoke significant (p<0.05) dose-dependent release in free glycerol compared to untreated control. These effects were much larger compared the results obtained in the absence of BNF (See figure 4.2 for comparison).

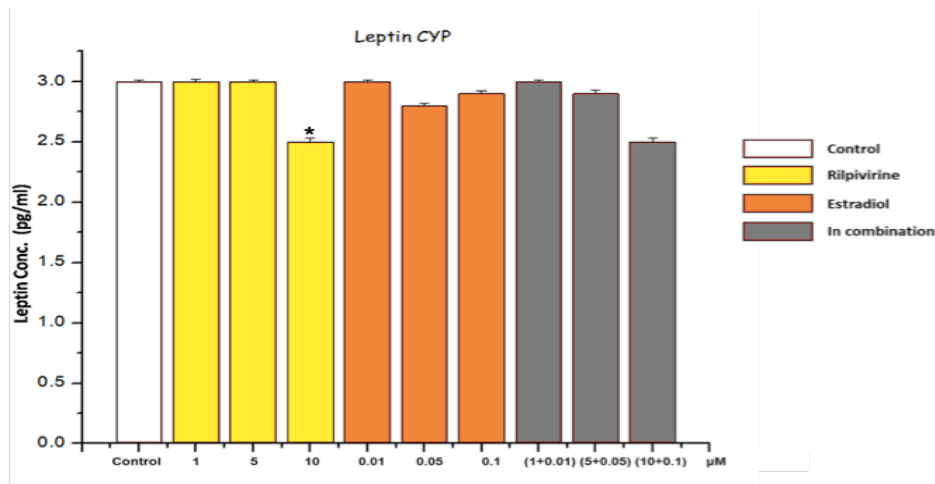


Figure 4.8: Bar charts showing the effect of different concentrations of rilpivirine or estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release of leptin by human adipocytes in culture for 10 days with the drugs. Values are means \pm SD; n=6; *p<0.05.

Figure 5.8 shows the effect of different concentrations of rilpivirine or estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release of leptin by human adipocytes in culture for 10 days with the drugs. The results show that only 10 μ M rilpivirine alone and 10 μ M rilpivirine in combination with 0.1 μ M estradiol with BNF can evoke significant (p<0.05) decreases in leptin compared to untreated control.

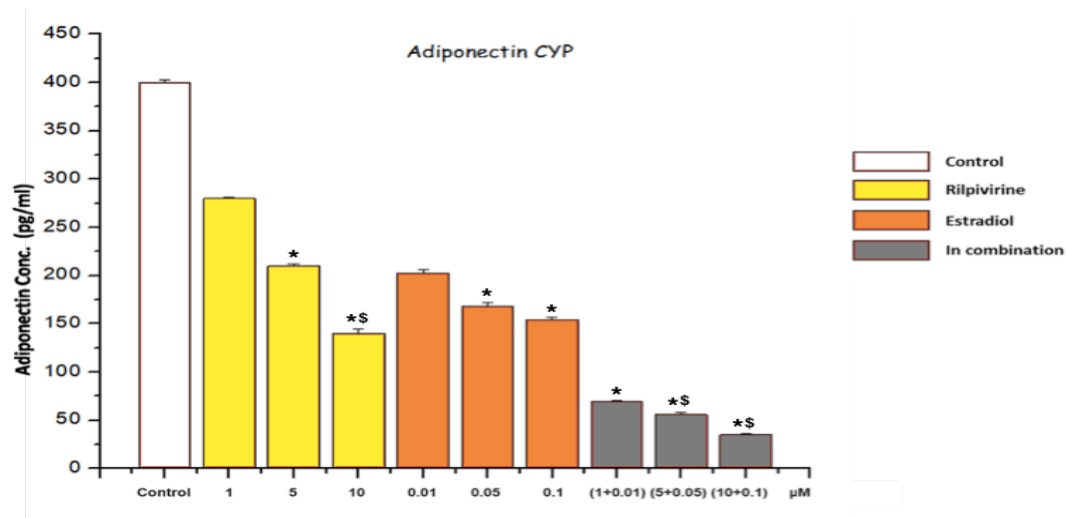


Figure 4.9: Bar graphs showing the effect of different concentrations of rilpivirine and estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release

of adiponectin by human adipocytes in culture for 10 days with the drugs. Values are means \pm SD; n=6; *p<0.05 for all drug treatments compared to untreated control.

Figure 4.9 shows the effect of different concentrations of rilpivirine and estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release of adiponectin by human adipocytes in culture for 10 days with the drugs. The results show significant (p<0.05) decreases in adiponectin release for all drug treatments compared to untreated control. The decrease in adiponectin level was dose dependent, but much more marked in the presence of BNF.

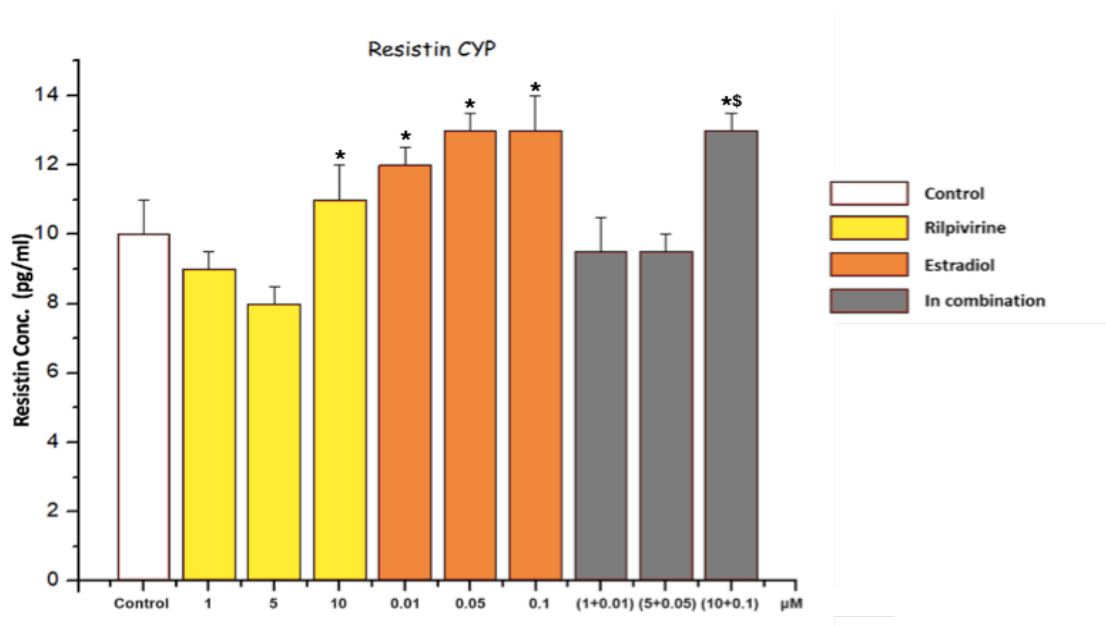


Figure 4.10: Bar graphs showing the effect of rilpivirine and estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release of resistin from human adipocytes in culture for all the drugs. Values are means \pm SD; n=6; *p<0.05for drug treated cells untreated control cells.

Figure 4.10 shows the effect of rilpivirine and estradiol either alone or in combination and in the presence of 200 μ M of BNF on the release of resistin from human adipocytes in culture for all drugs. The results show a significant (p<0.05) release in resistin for 10 μ M rilpivirine, all concentrations of estradiol and rilpivirine (10 μ M) in combination with estradiol (0.1 μ M) and BNF compared to untreated control.

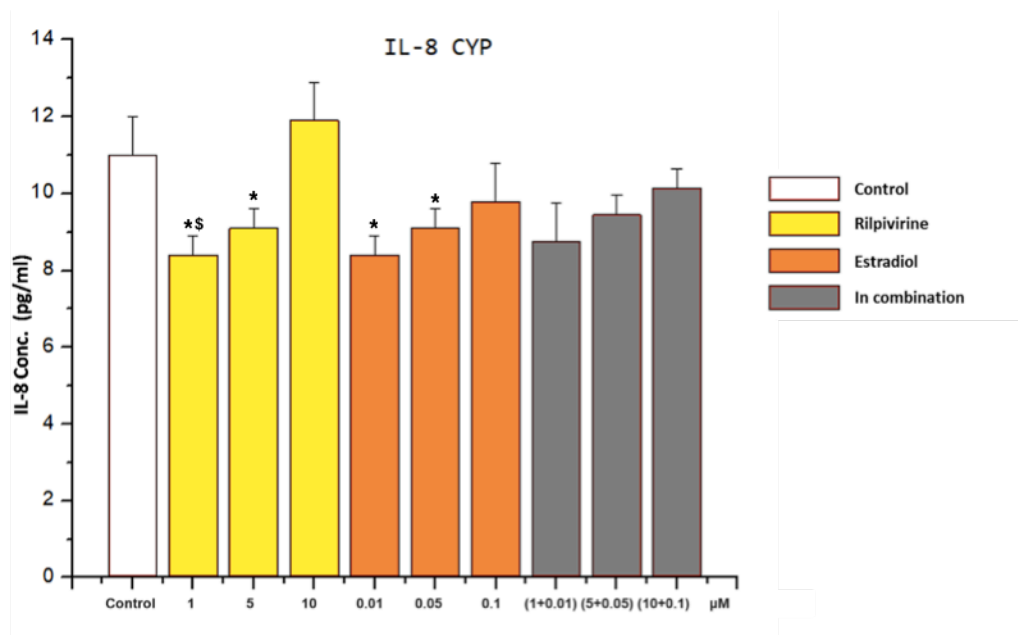


Figure 4.11: Bar charts showing the effect rilpivirine and estradiol either alone or in combination and in the presence of 200 μM of BNF on the release of interleukin-8 from human adipocytes in culture for 10 days with all the drugs. Values are mean ± SD; n=6; *p<0.05 for 1 μM and 5 rilpivirine, 0.01 μM and 0.05 μM estradiol compared to untreated cells.

Figure 4.11 shows the effect rilpivirine and estradiol either alone or in combination and in the presence of 200 μM of BNF on the release of IL-8 from human adipocytes in culture for 10 days with all the drugs. The results show a significant (p<0.05) decrease in the release of IL-8 for 1 μM and 5 μM rilpivirine, 0.01 μM and 0.05 μM estradiol compared to untreated cells.

4.5 Discussion

The results of this study have demonstrated marked morphological changes in the adipocytes in culture over time. The cells were observed daily as they were proceeding through the foregoing stages of differentiation. Microphotographs were taken on day 0, day 5 and day 10 for comparison. The results show that the shape of the cells changed from the spindle- shaped fibroblast to the nearly spherical form of a mature adipose cell. There was an increasing accumulation of lipid, first as small inclusions concentrated towards one pole of the cell and then finally as large lipid masses in the central region of the cell.

Viability of the cells were assessed on 0, 5 and 10th day of the adipocyte maturation process by employing lactate dehydrogenase as a marker to monitor cell death. Increasing concentrations of rilpivirine (1 μM, 5 μM and 10 μM) and estradiol (0.01 μM, 0.05 μM and 0.1 μM) were applied

individually and in combination. The results clearly show that neither rilpivirine nor estradiol either alone or in combination either with or without BNF had any deleterious effects on the viability of adipocytes compared to untreated cells. There was no significant release of LDH in either stimulated or treated cells as compared to untreated cells. The viability of cells at all stages of adipogenesis, as measured by the lactate dehydrogenase assay did not show any significant difference as compared to the control. Therefore, all these concentrations were determined as optimal for future experimental work. These results are in agreement with a previous study (Díaz-Delfín et al., 2012).

Data from several previous studies have revealed that anti-retroviral and estrogen therapy can induce lipid alterations by activating lipolysis and thereby, enhancing the levels of free fatty acid and free glycerol in adipocyte cell cultures (Ranganathan et al., Kern, 2002; Jones et al., 2005; Mallon, 2007).

The results of this study show an increasing trend in the lipolysis process in human adipocytes. The release of glycerol was dose-dependent with both rilpivirine and estradiol. Concomitant treatment of two drugs at all doses did not show any attenuation or potentiating effect on glycerol release, but only a small additive effect compared to the effect of either rilpivirine or estradiol alone. The observed results could be attributed to impaired GLUT4 translocation and are in keeping with another study that assessed the effect of nelfinavir on glucose uptake and lipolysis in differentiated 3T3-L1 adipocytes. An 18-h exposure to nelfinavir resulted in an impaired insulin-stimulated glucose uptake and activation of basal lipolysis with as low as 5 $\mu\text{mol/l}$ nelfinavir (Rudich et al., 2001). Another study conducted in vivo showed that exposure to high concentrations of estradiol led to region-and pathway-dependent modulation of subcutaneous adipose tissue lipolysis in pre-menopausal women (Gavin et al., 2013).

Leptin has been reported to be related to adiposity and it increases in proportion to decreasing adipose tissue stores. It is of much interest in anti-retroviral and estrogen-based therapies where abnormalities of body fat distribution are characteristic features. A number of in vitro studies have demonstrated a reduction in leptin secretion when human adipocytes were treated with ARVs (Ambati et al., 2007; Díaz-Delfín et al., 2011). In this study, no significant release in leptin level were observed when adipocytes were treated with either all concentrations of estradiol or low doses of rilpivirine compared to a higher dose of 10 μM which elicited a significant decrease in leptin secretion compared to untreated cells. Similar results were obtained with the highest dose of each drug when they were combined. Previous in vivo and in

vitro studies reported that estrogens can elevate the level of leptin (Ainslie et al., 2001; Geber et al., 2012). However, some reports have indicated either a nil or a reverse relationship between estrogens and leptin release (Rechberger et al., 1999; Xing et al., 2015).

Adiponectin, an insulin-sensitizing adipokine, is induced during adipocyte differentiation (Scherer et al., 1995). Reduced levels of adiponectin have been reported by various studies in adipocytes treated with very high doses of ARV drugs. In the present study, except for at a low concentration (1 μ M) of rilpivirine alone, all the other drug treatment doses elicited significant effects on the release of adiponectin levels from human adipocytes. Combining rilpivirine with estradiol produced a much larger decrease in the release of adiponectin compared to their individual concentrations alone. Adiponectin is known to enhance insulin-mediated glucose uptake in peripheral tissues (i.e., skeletal muscle) and in sensitizing liver to insulin-dependent suppression of glucose output (Kadowaki et al., 2006). This reduction may be considered as a major contributor to insulin resistance (IR). In this study, reduced levels of adiponectin were observed when cells were treated with increasing doses of 17 β -estradiol. The present results are in agreement with a recent study that reported dramatically suppressed levels of adiponectin concentration when pre-adipocytes were exposed to increasing levels of 17 β -estradiol (Pektaş et al., 2015).

Originally, resistin was thought to be a link between diabetes and obesity. A single nucleotide polymorphism in the resistin gene has been associated with elevated lipids, insulin resistance and body composition changes among HIV-infected individuals on anti-retroviral therapies (Steppan et al., 2001). In another study, greater plasma resistin levels were observed in HIV patients compared with uninfected controls (Escoté et al., 2011). However, other workers have found no association between serum resistin and fat redistribution, and metabolic profile in HIV-infected patients (Barb et al., 2005; Arama et al., 2014). The physiological role of resistin, therefore, is not fully understood and needs further studies. It is proposed to be an important peptide bringing about insulin resistance and obesity, although it is still debatable (Lappas et al., 2005). In the present study, the levels of resistin were comparable to that of control and there was no significant difference for pre-adipocytes treated individually with rilpivirine at low doses. Only a slight increase in the resistin level was observed at 10 μ M, indicating that rilpivirine may contribute to the fat re-distribution but only at high dosage. A positive correlation in estrogens and resistin levels has been demonstrated and resistin concentrations were found to be conspicuously elevated by 17 β -estradiol (Chen et al., 2006; Pektaş et al., 2015). The present

study showed enhanced levels of resistin in adipose cells treated with different concentrations of 17 β -estradiol as compared to control. Combination of rilpivirine and estradiol did not show any increase in resistin level for the lowest and median doses used in the study. However, an increase in the resistin concentrations was observed at the highest dosage of combination treatment (1 μ M rilpivirine combined with 0.1 μ M estradiol).

Increased serum IL-8 levels have been previously reported in HIV patients with impaired glucose tolerance (Reeds et al., 2006), and this is consistent with findings in T2DM (Zozulinska et al., 1999), obesity and non-alcoholic fatty liver disease (Jarrar et al., 2008). Chronic inflammatory state in HIV-infection may be associated with elevated fasting serum level of IL-8. Higher serum IL-8 may also reflect increased production and release from the larger adipose depots and may have an influence on lipid metabolism along with its effect on insulin resistance. Elevated levels of IL-8 have been observed in human subcutaneous and visceral adipose tissue obtained from HIV-infected people with fat redistribution and insulin resistance (Lihn et al., 2003). In the present study, IL-8 levels increased with an increase in the concentrations of rilpivirine. High concentrations of IL-8 were observed when the cells were treated with 10 μ M of rilpivirine indicating its role in fat re-distribution. The present findings are in agreement with another study (Díaz-Delfín et al., 2012), which reported that rilpivirine did not have any effect on the release of IL-8 at concentrations up to 4 μ M, but instead, it produced a marked and significant elevation only at 10 μ M. Compared to rilpivirine, estradiol evoked a dose-dependent increase in the release of IL-8 from adipose cells. Enhanced levels of IL-8 were observed when the pre-adipocytes were treated with increasing concentrations of estradiol. Moreover, increasing release in the level of IL-8 was observed when adipocytes were treated with a combination of rilpivirine and estradiol suggesting a major contribution of estradiol in eliciting the release of IL-8.

In the second series of experiments human adipocytes were pretreated with BNF in the absence and presence of either rilpivirine, estradiol or when the two drugs were combined for a period of 10 days. The results reveal elevated glycerol level with BNF alone. When BNF was combined with either rilpivirine, estradiol or both drugs, there was enhanced release of glycerol in a dose-dependent manner. BNF seems to be potentiating the effect of either rilpivirine or estradiol on glycerol release (enhanced lipolysis) from human adipocytes. In contrast, BNF had no significant effect on the release of leptin from human adipocytes when combined with either rilpivirine, estradiol or with both. In this series, BNF also enhanced the decrease in adiponectin release

from human adipocytes in the presence of either rilpivirine, estradiol or when the two drugs were combined compared to the results obtained in the absence of BNF. In contrast, in the presence of BNF, individual treatment of adipose cells with either rilpivirine or estradiol alone or combined did not seem to have any significant effect on the resistin and IL-8 releases for all the doses used in the study compared to the results obtained in the absence of BNF. In fact, BNF seemed to induce a reduction in the levels of resistin and IL-8 as compared to levels in the absence of BNF.

In conclusion, the results of this study show that a combination of rilpivirine and 17 β -estradiol is completely non-toxic to pre-adipocytes at most concentrations tested. During the 10-day exposure to the drugs, only estradiol produced a modest cytotoxic effect at the highest concentrations tested. No profound deleterious effect was observed on adipose tissue development or the endocrine function of adipose tissue (adipokine and cytokine release) in treated 3T3-L1 human pre-adipocytes. However, caution should be maintained considering that other transcriptional factors play a crucial role in overall alterations of lipid metabolism. BNF does not seem to significantly interact with rilpivirine and 17 β -estradiol except for the release of adiponectin. Moreover, to establish and elucidate a significant effect of these drugs on the adipogenic process, further studies at gene expression levels and involving the markers of oxidative stress are required.

Chapter 5

Effect of rilpivirine on human subcutaneous adipose cells and its nutritional management using quercetin.

5.1 Introduction

The widespread use of highly active antiretroviral treatment (HAART) has radically transformed the prognosis of HIV infected patients. Anti-retroviral therapy for HIV infection has transformed it from a terminal illness to a chronic manageable condition (Bailey et al., 2008; Palella et al., 1998). However, long-term treatment of HIV 1-infected patients can result in the appearance of several secondary effects, the most frequent being the so-called 'HIV-infection, HAART treatment-associated lipo-dystrophy syndrome (HALS) (Montessori et al., 2004).

Lipo-dystrophy is associated with both morphological (lipo-atrophy, lipo-hypertrophy) and metabolic (dyslipidaemia, glucose intolerance, hypertension, endothelial dysfunction and atherosclerosis) alterations, which may occur singly or in combination. Moreover, they are associated with an increased risk of diabetes and cardiovascular diseases. HAART-induced adipocyte inflammation, oxidative stress and macrophage infiltration, as well as altered adipocyte function are considered as major contributors in the development of HALS. The adipocyte, therefore, represents a plausible target for treatment (Boss, 2003). Pharmacological and surgical treatment interventions have shown some effect. However, their use is associated with numerous adverse side effects and complications (Slaven et al., 2003; Rasmussen et al., 2011; Liu et al., 2012; Grulich et al., 2007). Targeted lifestyle interventions may provide a useful alternative for managing HALS owing to their safety and tolerability.

The WHO recommends that nutrition interventions should be part of all HIV/AIDS control and treatment programme because adequate diet and nutrition can improve adherence to anti-retroviral therapy (ART) and its effectiveness, as well as help tackling metabolic abnormalities in people living with HIV/AIDS (World Health Organization, 2003). In 1989, the World Health Organization (WHO) had declared the necessity to assess ethno-medicines and other natural products for the management of HIV/AIDS. To quote: "In this context, there is need to evaluate those elements of traditional medicine, particularly medicinal plants and other natural products that might yield effective and affordable therapeutic agents. This will require a systematic approach" (World Health Organization, 1989a,b). Moreover, because they are fairly not as expensive and have less side effects, phyto-medicines are retrieving patient approval (Vermani et al., 2002; Short, 2006). The use of complementary and alternative medicines, including herbal medicines, is common among individuals with HIV. The reported prevalence of herbal remedies use by HIV-infected individuals in the United States varies between 12% and 27.2 % (Abbot et al., 1997; KM et al., 1998; Sparber et al., 2000). Surveys have revealed that nearly 67% of HIV-

infected patients receiving ARV therapy also take a natural health product (NHP)(Gore-Felton et al., 2003), of which the most common are vitamin C (63%), multivitamins (54%), vitamin E (53 %), and garlic (53%); vitamin B12, beta-carotene, acidophilus, vitamin B6, zinc, ginseng, selenium, *Echinacea* species, vitamin A, aloe, folate, Chinese herbs, marijuana, goldenseal, and coenzyme Q10 which were reportedly taken by 20%–50% of patients (Standish et al., 2001). The major reasons reported by HIV-infected patients for using these complementary alternative medicines modalities are a perceived additional efficacy, an increase in quality of life, a reduction of adverse effects of ARV therapy, and a feeling of control (Duggan et al., 2001).

Rilpivirine (Trade name: Edurant), is a prescription medicine approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV infection in adults and children 12 years of age and older who have never taken HIV medicines before and who have a viral load (number of HIV RNA copies /mL of blood) of 100,000 copies/mL or less at the start of treatment. (Schrijvers et al.,2011). It belongs to a class (group) of HIV drugs called non-nucleoside reverse transcriptase inhibitors (NNRTIs) and acts by binding directly to the enzyme at a site different from the nucleoside binding component, thereby causing an allosteric inhibition of the transcriptase.

Quercetin, a plant pigment, is a potent antioxidant flavonoid and more specifically a flavonol, found mostly in onions, grapes, berries, cherries, broccoli, and citrus fruits. It is a versatile antioxidant known to possess protective abilities against tissue injury induced by various drug toxicities. It is an important bioflavonoid known for its anti-inflammatory, antihypertensive, vasodilator effects, anti-obesity, anti-hyper-cholesterolemic and anti-atherosclerotic activities (Sultana and Anwar, 2008; Salvamani et al., 2014).

Aims: The main aims of the present study were to determine the metabolic alterations caused by rilpivirine and also to assess the nutritional effect of quercetin on preventing and improving these metabolic changes.

5.2 Materials and methods

As described in chapter 2.

5.3 Results

5.3.1 Measurement of cyto-toxicity using lactate dehydrogenase assay

The human subcutaneous pre-adipocytes were seeded at a density of 2×10^4 cells per well in 24-well plates and cultured until 90 % confluence in culture medium. The cells were then treated with various concentrations of rilpivirine or quercetin either alone and in combination for a period of 15 days. As an indicator of cytotoxicity, the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the culture supernatant of the control as well as treated differentiating adipose cells on day 3 and day 15 of culture. An optimized LDH test was used to quantify LDH activity in the medium of the treated differentiating adipose cells. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

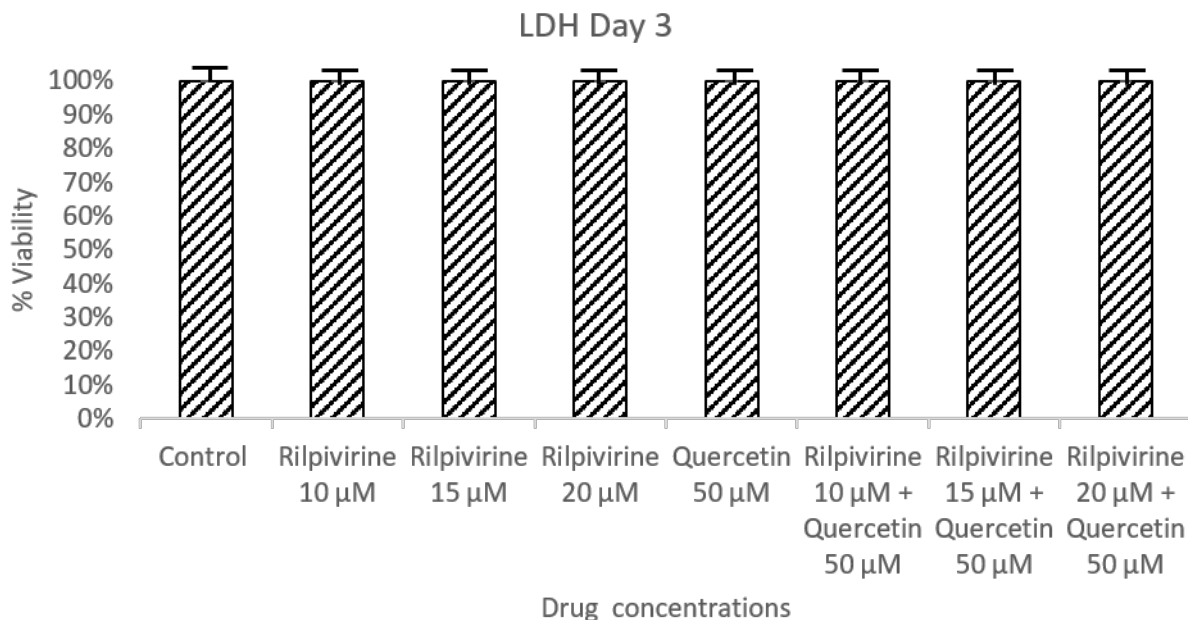


Figure 5.1: Bar graph showing the effect of rilpivirine or quercetin either alone or in combination on the cell viability as measured by LDH on day 3 of culture. Human subcutaneous adipose cells were treated with various concentrations of indicated drugs either alone and in combination. Cytotoxicity was expressed as the percentage of LDH activity in the medium as compared to control on day 3 of incubation. The results represent mean \pm SEM of 3 parallel cell culture experiments. * $p < 0.05$ compared to the control.

The results presented in figure 5.1 show no significant ($p>0.05$) changes in the viability of cells when treated with various concentrations of either rilpivirine or quercetin alone and in combination for day 3 of culture.

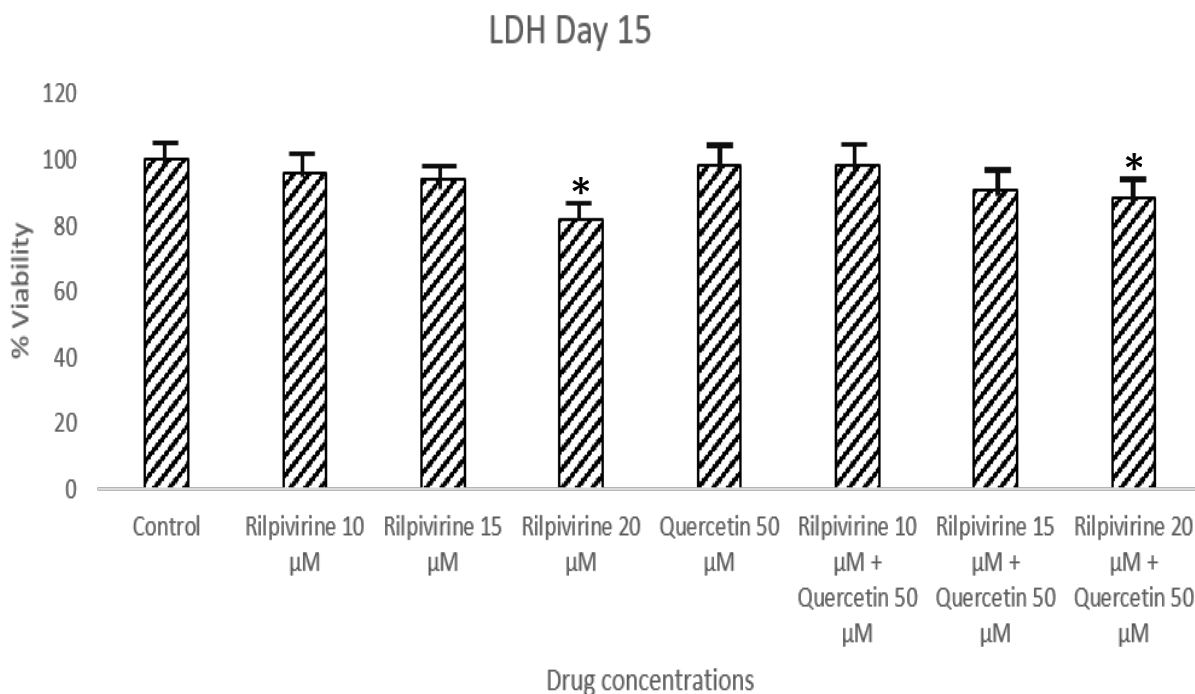
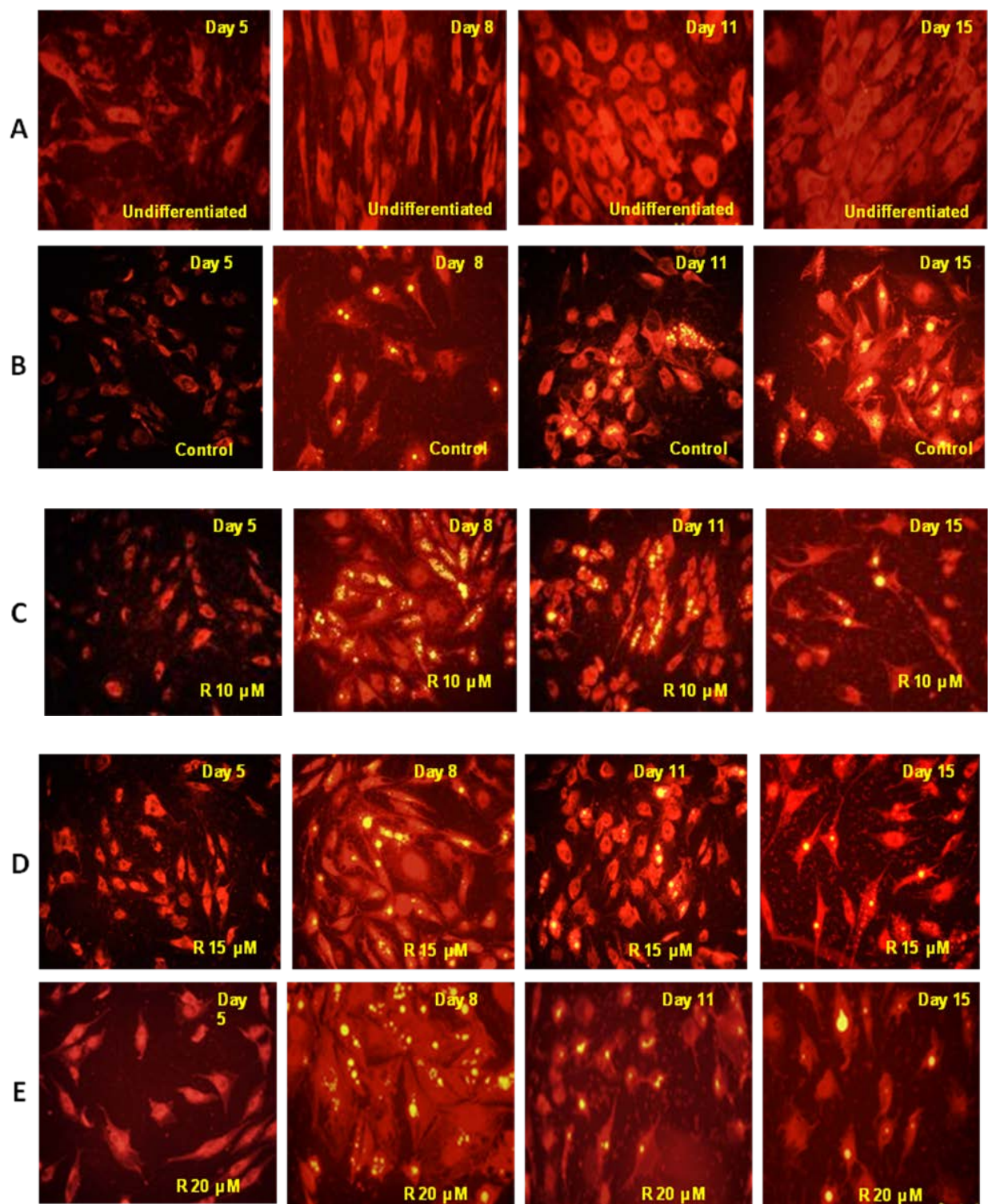


Figure 6.2: Bar graph showing the effect of rilpivirine or quercetin either alone or in combination on the cell viability as measured by LDH on day 15 of culture. Human subcutaneous adipose cells were treated with various concentrations of indicated drugs either alone and in combination. Cyto-toxicity was expressed as the percentage of LDH activity in the medium as compared to control on day 15 of incubation. The results represent mean \pm SEM of 3 parallel cell culture experiments. * $p<0.05$ compared to the control.

The results presented in figure 5.2 show that rilpivirine at lower concentrations (10 μ M and 15 μ M) and quercetin at 50 μ M did not alter the viability rate of the cells. However, at a concentration of 20 μ M, a slight and significant ($p<0.05$) decrease in the cell viability was observed indicating its effect at higher doses. A combination of rilpivirine and quercetin at drug concentration of 20 μ M and 50 μ M respectively, also showed a significant ($p<0.05$) decrease.

5.3.2 Morphological Examination of Adipose cells by AdipoRed Staining



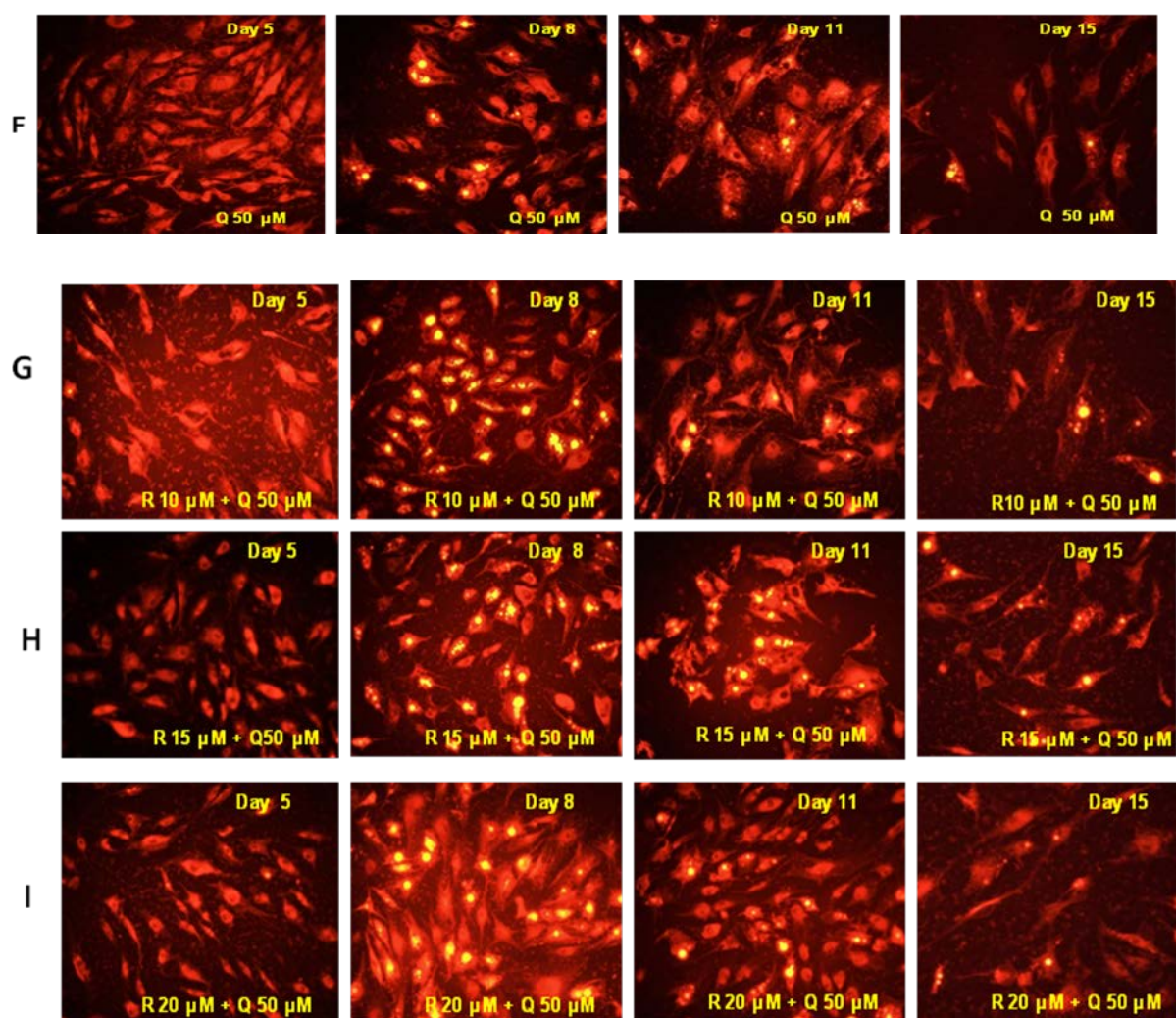


Figure 5.3: Original representative images (A-I) showing the progression of adipogenesis as indicated by lipid formation for human adipose cells in culture. Human subcutaneous adipose cells were differentiated and treated with different concentrations of either rilpivirine or quercetin individually and then in combination. Images were taken for the 5th, 8th, 11th and 15th day of cells in culture at 20x magnification using a fluorescence microscope. Staining was done using adipored to visualize lipid content. Images are typical of 3 such different experiments in duplicate. These images reveal the following:

- A). Undifferentiated and untreated (no drugs) adipose cells.
- B). Differentiated and untreated (no drugs) adipose cells.
- C). Adipocytes differentiated and treated with 10 μ M of rilpivirine.
- D). Adipocytes differentiated and treated with 15 μ M of rilpivirine.
- E). Adipocytes differentiated and treated with 20 μ M of rilpivirine.
- F). Adipocytes differentiated and treated with 50 μ M of quercetin.

G). Adipocytes were differentiated and treated with a combination of 10 μ M rilpivirine and 50 μ M quercetin.

H). Adipocytes differentiated and treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin.

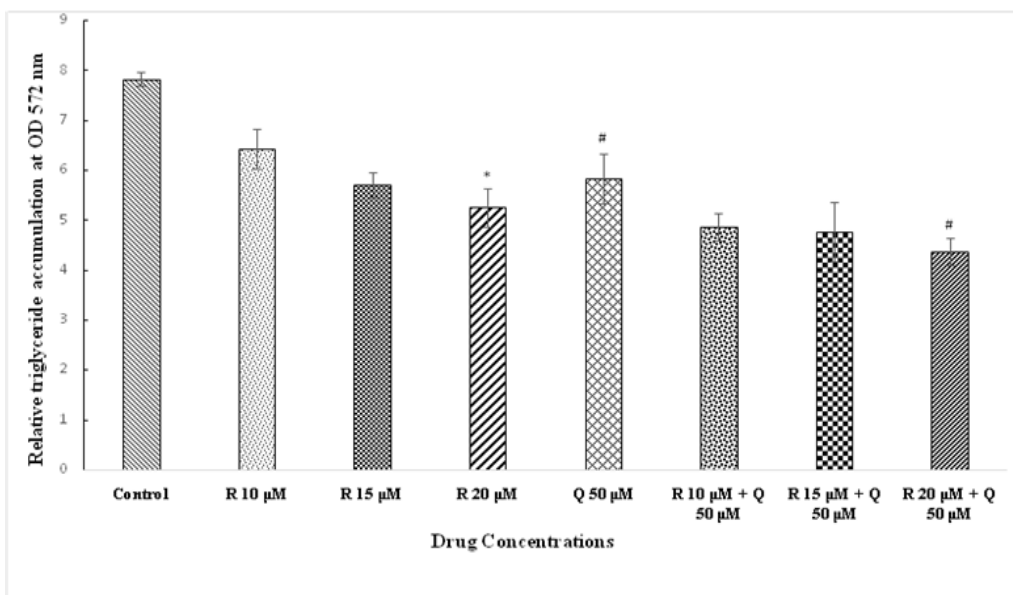
I). Adipocytes differentiated and treated with a combination of 20 μ M rilpivirine and 50 μ M quercetin.

Morphological data from figure 5.3 show the accumulation of triglyceride in pre-adipocytes cultured and differentiated in the presence/absence of treatment drugs either alone or in combination. Image A represents undifferentiated and untreated pre-adipocyte cells in culture. These cells appeared elongated, fibroblast-like, spindle shaped and showed no lipid accumulation. Image B represents the progression of pre-adipocyte cells into mature adipose cells differentiated for 15 days without any drug treatment (Control). The process of adipogenesis progressed in a time-dependent manner as evidenced by the increasing lipid droplets (yellow). On day 5, almost nil or very little amount of lipid droplets were observed. However, by day 8 the cells began to attain a rounder shape and became laden with lipids and had accumulated substantial triglyceride content. These lipid vacuoles were observed to accumulate more triglycerides and grow more in size and number on day 11 and 15 in culture. Images C, D and E represent pre-adipocyte cells treated with a dosage concentration of 10 μ M, 15 μ M and 20 μ M of rilpivirine respectively over a period of 15 days. It was observed that rilpivirine inhibited the accumulation of triglycerides in a dose- and time- dependent manner. The formation of lipid droplets (yellow) decreased with increasing dosage and time in culture. Images F represents pre-adipocytes treated with 50 μ M of quercetin. Pre-adipocytes treated with quercetin were shown to inhibit triglyceride accumulation with increasing number of days in culture. The number of lipid droplets decreased from day 8 until day 15 in culture. Images G, H and I represent pre-adipocyte cells treated with a combination of 10 μ M, 15 μ M, 20 μ M of estradiol and 50 μ M of quercetin, respectively. Adipose cells treated with these combinations of drugs showed attenuation in lipid formation in time- and dose- dependent manner. Day 8 of differentiation showed a substantial amount of triglyceride accumulation which almost became diminished until day 15.

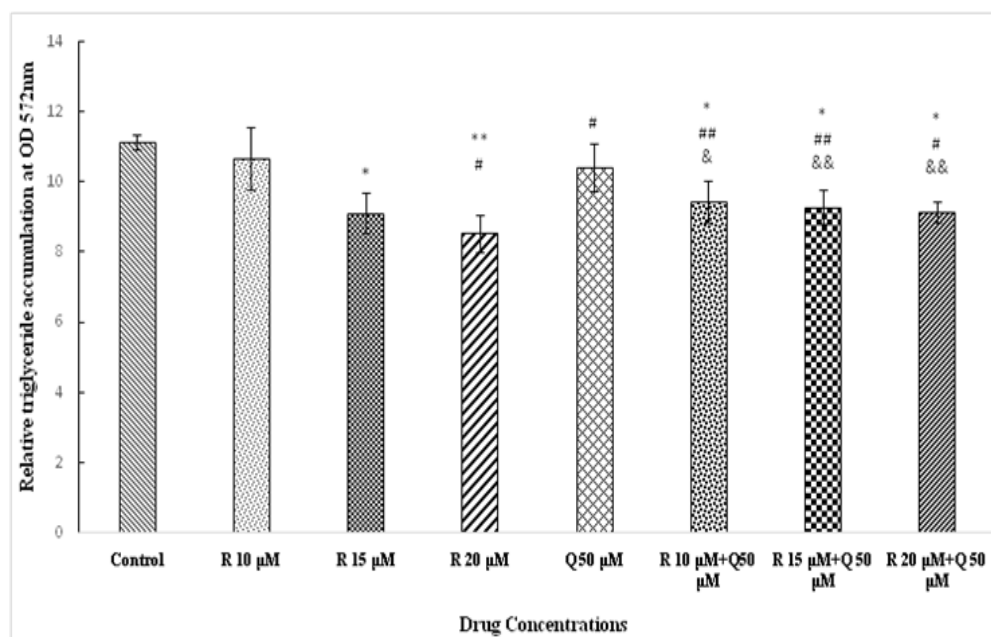
5.3.3 Quantitative measurement of triglycerides

Triglycerides accumulated over the differentiation period of 15 days were measured quantitatively by staining the cells with adipored reagent and the fluorescence was measured at OD value of 572 nm.

A
DAY 5



B
DAY 8



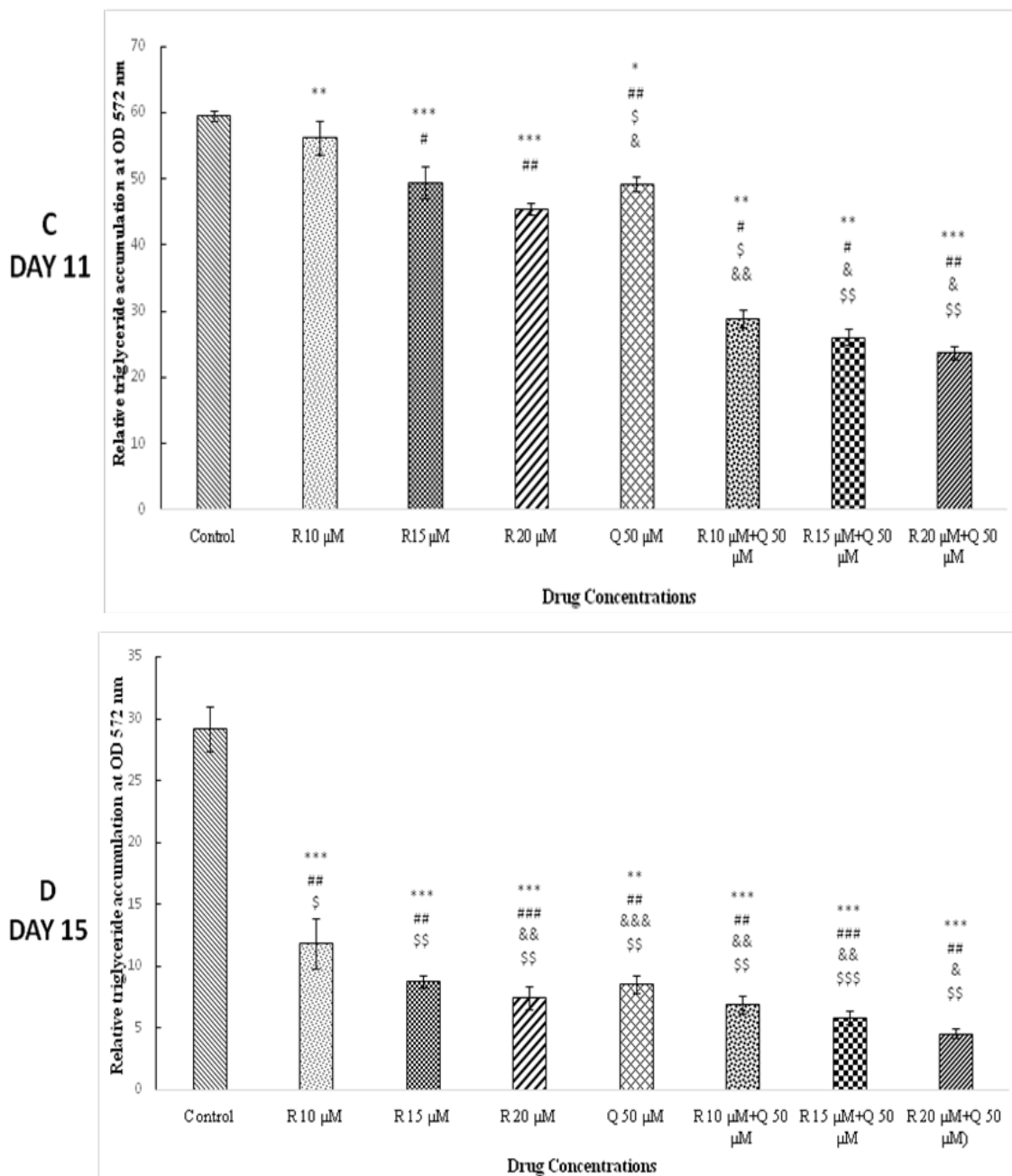


Figure 5.4 (A-D): Bar graphs showing the effect of rilpivirine and quercetin individually and then in combination on the accumulation of triglyceride by human adipocytes in culture. Human pre-adipocytes were differentiated in culture in the presence of indicated concentrations of rilpivirine and quercetin. Values represent concentration of triglyceride in cell supernatant for the 3rd, 5th, 8th and 11th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed

relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. #, \$, & indicate significance differences within the treatment groups.

Figure 5.4 (Images A-D) shows the progression of undifferentiated pre-adipocytes into differentiated mature adipose cells over a period of 15 days either in the presence or absence of indicated concentrations of treatment drugs. Adipose cells that were not treated with any drugs (control) showed a significant progression in the accumulation of triglycerides over 15 days in culture. Image A shows the accumulation of triglycerides on the 5th day of culture. Rilpivirine at concentrations of 10 and 15 μM did not induce any significant change in the lipid accumulation. However, a small but significant ($p < 0.05$) decrease was observed at a concentration of 20 μM of rilpivirine. Adipose cells treated with a dose of 50 μM of quercetin also evoked a slight reduction in the triglyceride levels. Combination of rilpivirine and quercetin decreased the triglyceride concentration in a dose dependent manner.

The results of triglyceride accumulation were more pronounced on day 8 and day 11 of culture (Image B and C). Rilpivirine at concentrations of 15 μM and 20 μM showed a significant ($p < 0.05$ and $p < 0.01$ for day 8 and $p < 0.01$ and $p < 0.001$ for day 11 respectively) decrease in triglyceride levels. Quercetin also inhibited the triglyceride level significantly ($p < 0.001$). When the two drugs were combined, significant reduction in triglyceride were measured. Triglyceride levels for day 15 (Image D) showed substantial reductions ($p < 0.001$) for adipose cells treated with either rilpivirine or quercetin alone and in combination. This reduction was more prominent and significant when the two drugs were combined together.

Overall, the results show that adipose cells treated with either rilpivirine or quercetin alone and in combination significantly ($p < 0.05$ for day 8 and $p < 0.001$ for day 11 and 15, respectively) inhibited lipid formation and attenuated triglyceride accumulation in a dose and time dependent manner. This reduction was more prominent when adipose cells were treated with a combination of estradiol and quercetin.

5.3.4 Quantitative measurement of inflammatory adipocytokines

Human subcutaneous adipose cells were cultured and differentiated over a period of 8 days in the presence of either rilpivirine or quercetin alone and in combination using maximal dose of each drug. Supernatant was collected on the 8th day of culture and the released adipocytokines (Adiponectin, leptin, resistin, IL-8) were measured quantitatively using commercially available kits.

5.3.4.1 Quantitative measurement of adiponectin

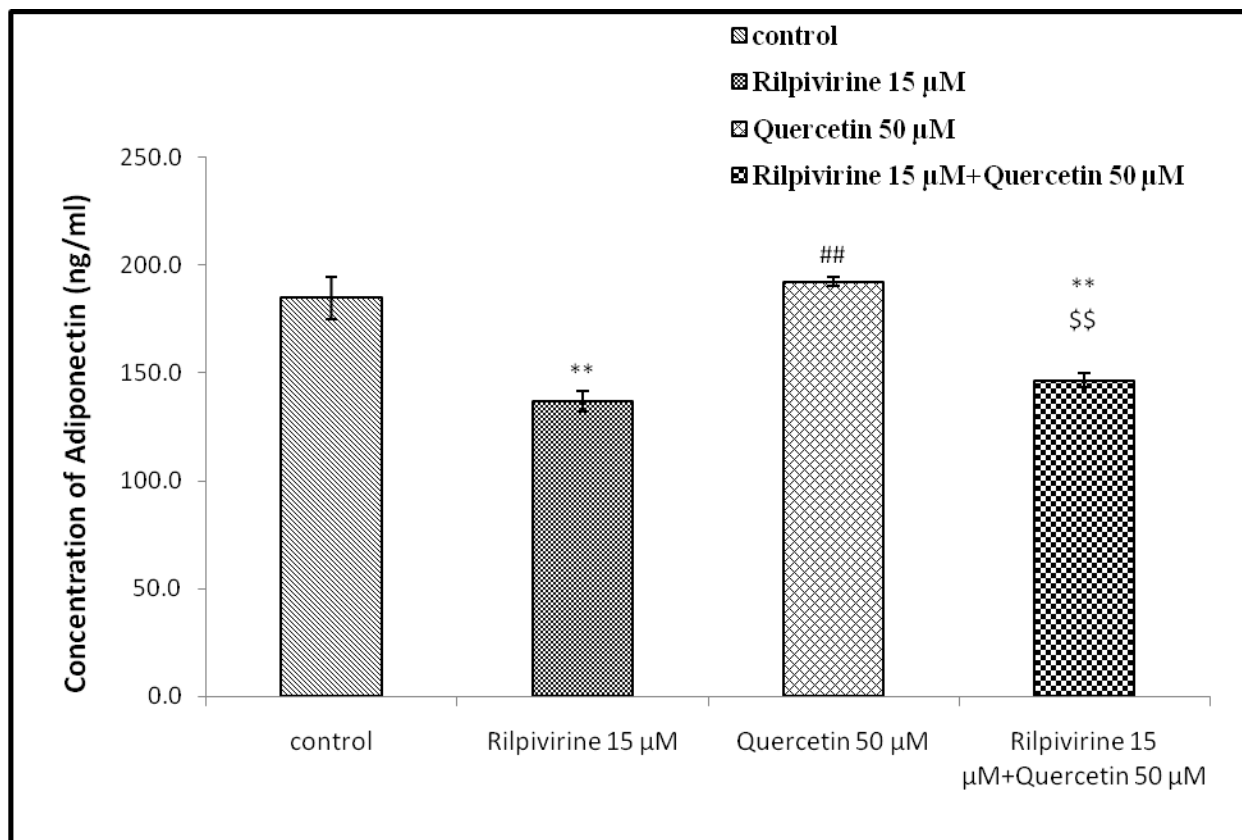


Figure 5.5: Bar graphs showing the effect of rilpivirine and quercetin individually and then in combination on the release of adiponectin by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin). Values represent concentrations of released adiponectin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean ± S.D from triplicate set of experiments expressed relative to values from untreated control cells; *p<0.05 **p<0.01 ***p<0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results in figure 5.5 show marked significant reduction in the adiponectin concentration levels as compared to control when treated with rilpivirine alone (p<0.01). Significant differences were observed in the adipocytes treated with a combination of rilpivirine and quercetin when compared with control (p< 0.01) as well as with quercetin alone (p< 0.01). Quercetin alone showed significant (p<0.01) increase in adiponectin levels when compared to the untreated or control group.

5.3.4.2 Quantitative measurement of leptin

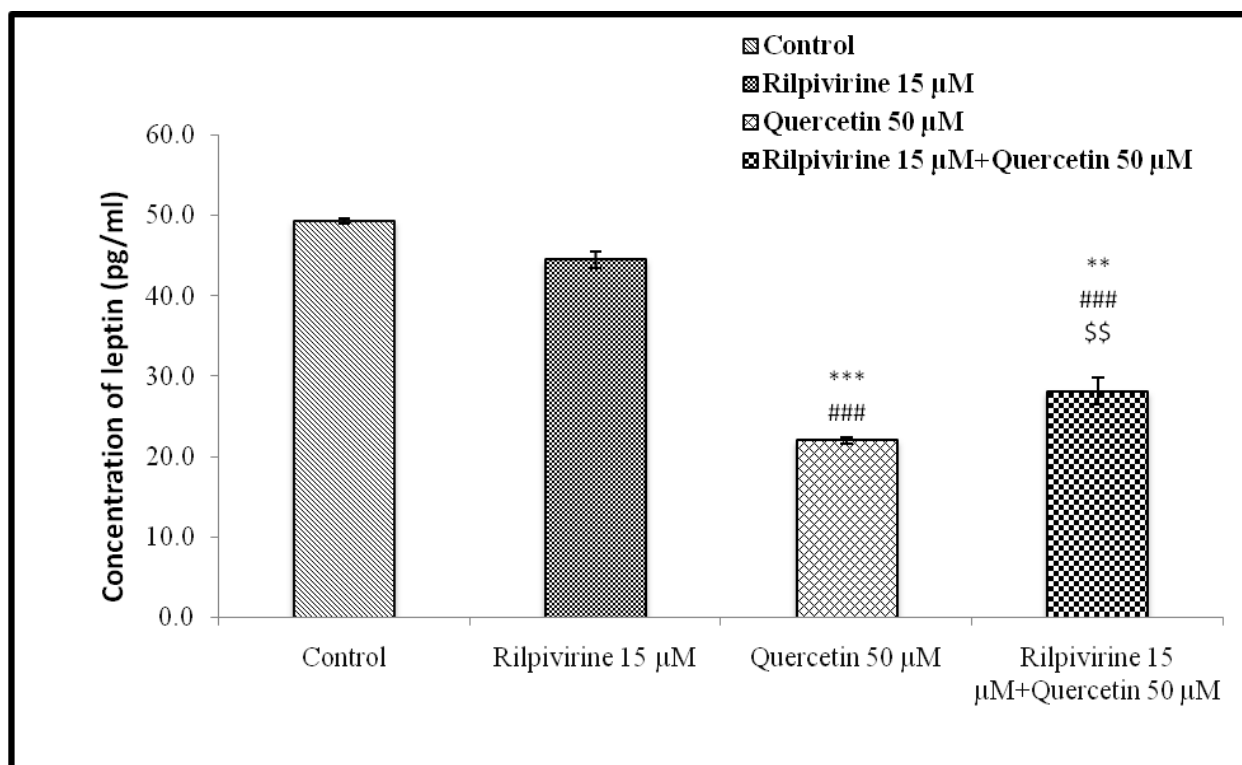


Figure 5.6: Bar charts showing the effect of rilpivirine and quercetin individually and then in combination on the release of leptin by human adipocytes in culture. Human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drug (15 µM rilpivirine and 50 µM quercetin). Values represent concentrations of leptin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and the results are presented as means \pm SD from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results in figure 5.5 show small and insignificant decrease in the adipocytes treated with rilpivirine alone. Treatment with quercetin alone showed significant (p <0.001) decrease in leptin levels when compared to rilpivirine alone. Adipose cells treated with a combination of rilpivirine and quercetin showed significant (p <0.01) decrease in the leptin levels as compared to control.

5.3.4.3 Quantitative measurement of Resistin

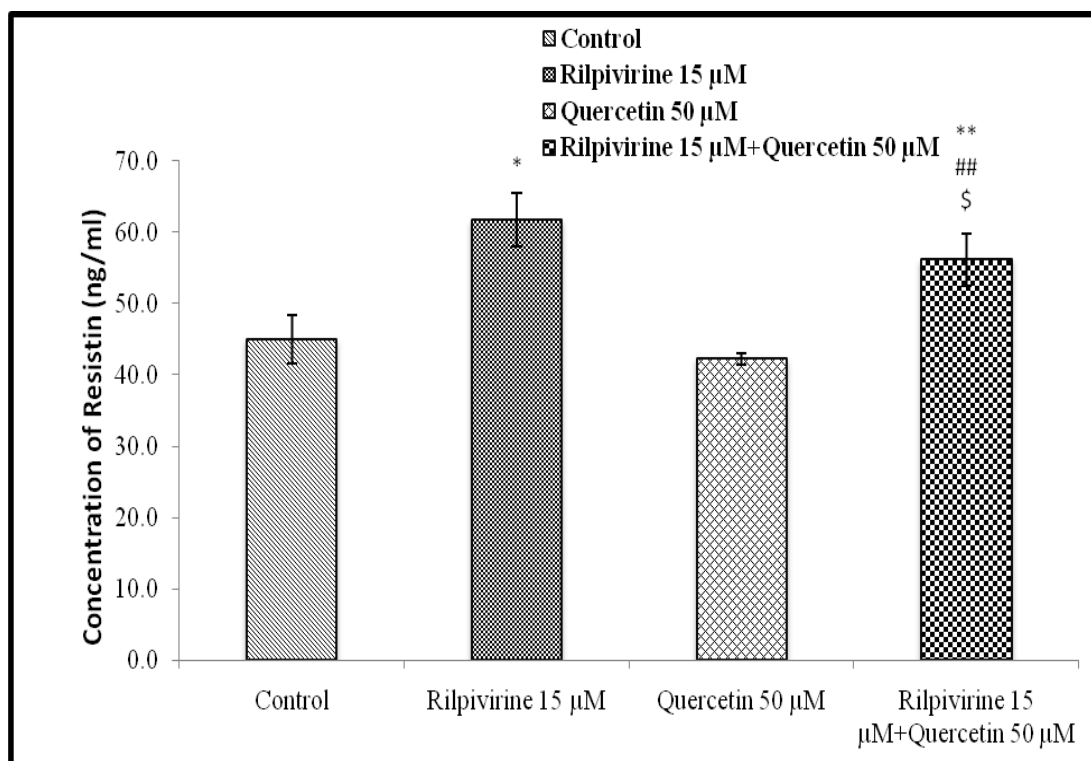


Figure 5.7: Bar charts showing the effect of rilpivirine and quercetin individually and then in combination on the release of resistin by human adipocytes in culture. Human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin). Values represent concentrations of resistin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean ± SD from triplicate set of experiments expressed relative to values from untreated control cells; *p<0.05 **p<0.01 ***p<0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The data in figure 5.7 show significant increase in resistin levels of adipose cells when treated with rilpivirine alone (p<0.05) or in combination with quercetin (p<0.01) as compared to control. The results also show that 50 µM of quercetin alone elicited only a small increase but insignificant release of resistin as compared to control.

5.3.4.4 Quantitative measurement of IL-8

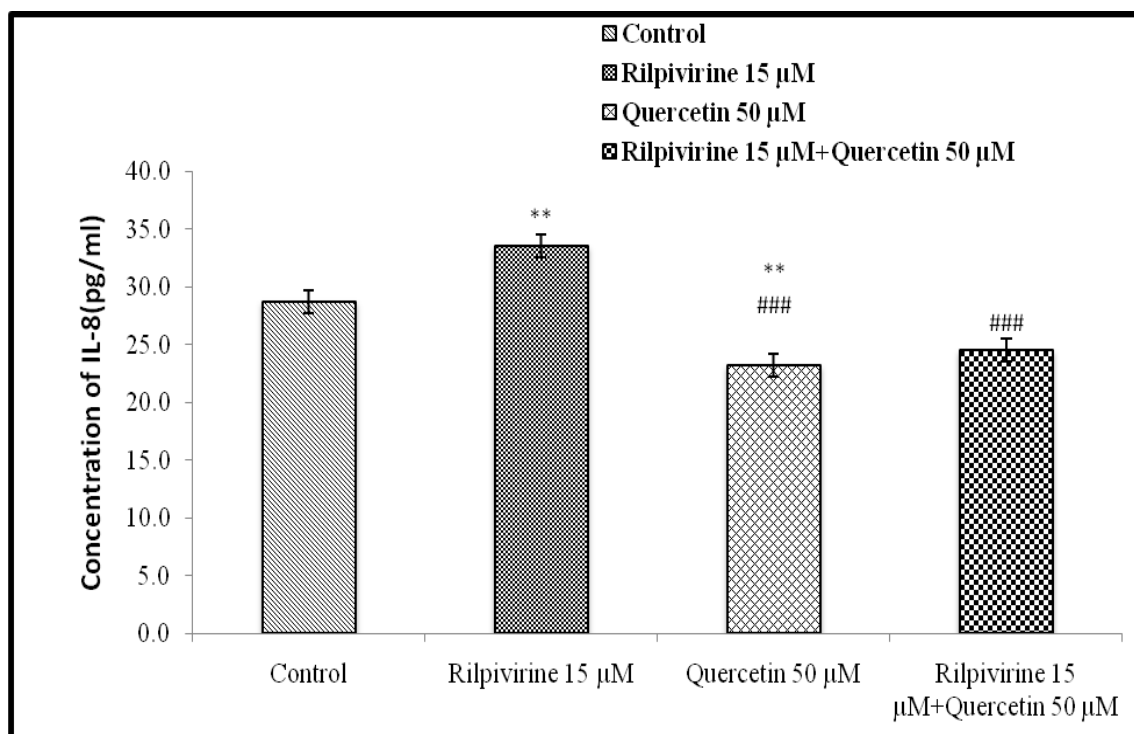


Figure 5.8: Bar charts showing the effect of rilpivirine and quercetin individually and then in combination on the release of Interleukin-8 by human adipocytes in culture. Human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin). Values represent concentration of adiponectin in the cell culture medium for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA with levene's test for equality of variances and are presented as mean \pm SD from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control.

The results in figure 5.8 show significant increase in the levels of IL-8 for adipose cells treated with rilpivirine ($p < 0.01$) as compared to control. Treatment with quercetin alone showed a small reduction in IL-8 levels. However, when the two drugs were combined, there was a significant ($p < 0.001$) reduction in IL-8 concentration in the cell supernatant.

5.3.5 Quantitative measurement of Oxidative stress markers

Markers of oxidative stress (Nitric oxide, Superoxide dismutase, Glutathione and Catalase) were measured quantitatively using commercially available kits.

5.3.5.1 Quantitative measurement of Catalase activity

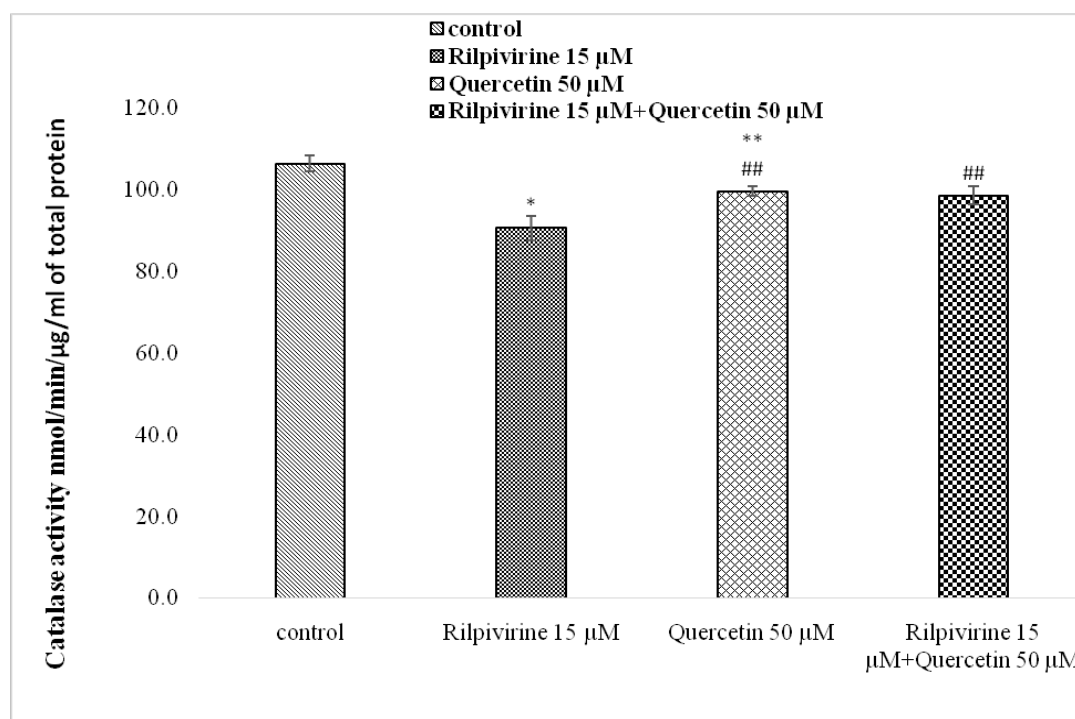


Figure 5.9: Bar charts showing the effect of rilpivirine and quercetin individually and then in combination on the catalase activity by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 μM rilpivirine and 50 μM quercetin). Values represent concentrations of catalase enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

Data from figure 5.9 show significant difference in the catalase activity for adipose cells treated with rilpivirine or quercetin alone ($p < 0.05$ and $p < 0.01$ respectively) as compared to control. The results also show significant differences ($p < 0.01$) among the adipose cells treated with quercetin alone. The results for combination of the two drugs showed values almost comparable to that of control.

5.3.5.2 Quantitative measurement of SOD activity

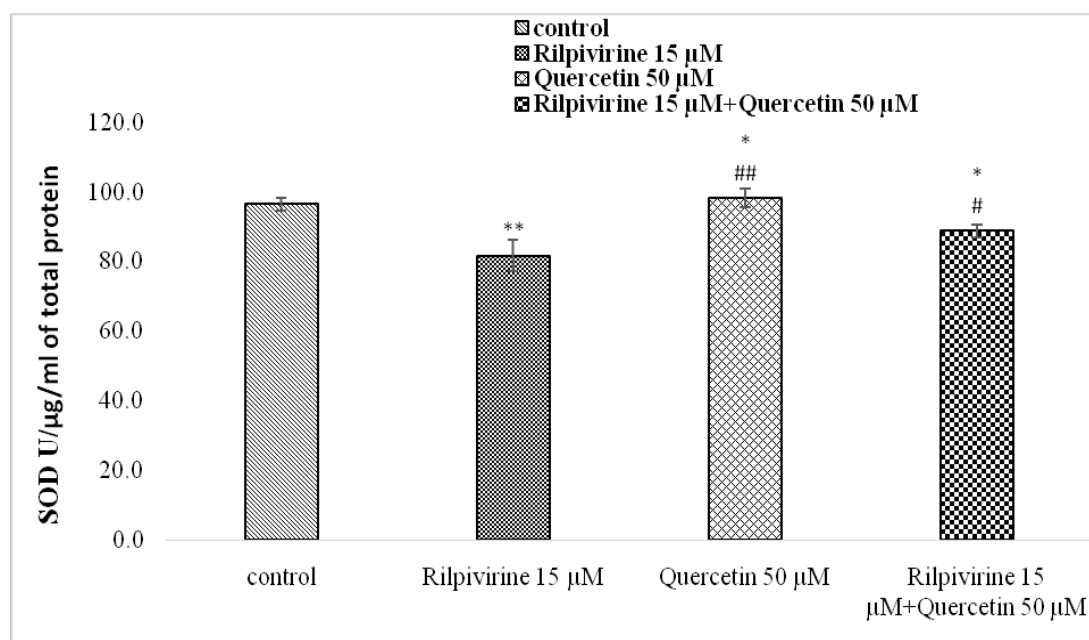


Figure 5.10: Bar graph showing the effect of rilpivirine and quercetin individually and then in combination on the Superoxide dismutase activity by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 μM rilpivirine and 50 μM quercetin). Values represent concentrations of superoxide dismutase enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA with for equality of variances and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

Data from figure 5.10 show significant decrease ($p < 0.01$) in SOD levels for adipose cells treated with rilpivirine alone. Adipose cells treated with quercetin alone showed a small but significant ($p < 0.05$) increase in the SOD activity. A combination of rilpivirine and quercetin also showed a small but significant ($p < 0.05$) increase as compared to adipose cells treated with rilpivirine alone.

5.3.5.3 Quantitative measurement of Nitric oxide

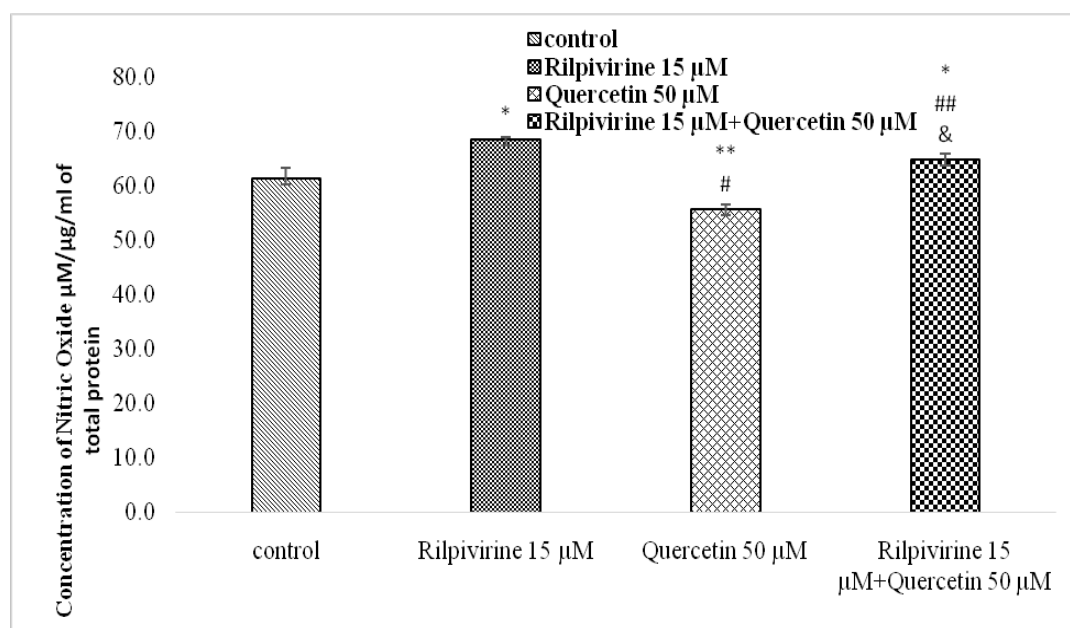


Figure 5.11: Bar charts showing the effect of rilpivirine and quercetin individually and then in combination on the concentration of nitric oxide by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin). Values represent concentrations of nitric oxide enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA with for equality of variances and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The results presented in figure 5.11 show significant differences in the treatment groups as compared to control. Treatment of adipose cells with rilpivirine alone elicited a marked increase ($p < 0.05$) in the NO levels as compared to control. Nitric oxide levels were significantly ($p < 0.01$) reduced in adipose cells treated with quercetin or its combination with rilpivirine ($p < 0.05$).

5.3.5.4 Quantitative measurement of Glutathione

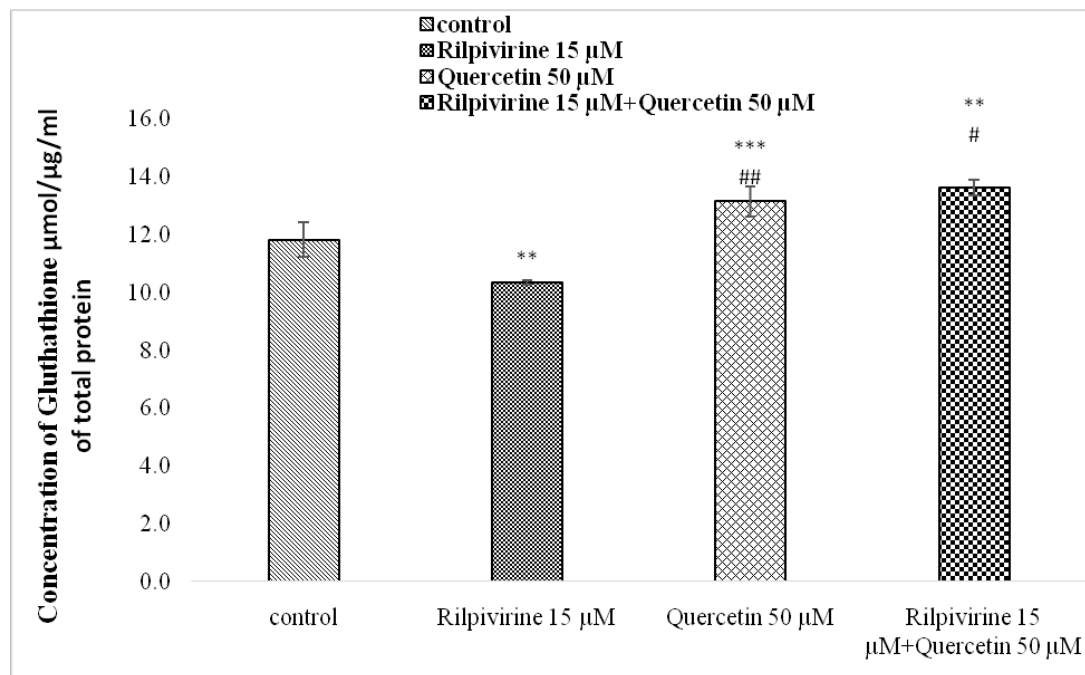


Figure 5.12: Bar graph showing the effect of rilpivirine and quercetin individually and then in combination on the concentration of glutathione by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 μM rilpivirine and 50 μM quercetin). Values represent concentrations of glutathione enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA with for equality of variances and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The results presented in figure 5.12 show significant decrease ($p < 0.01$) in glutathione activity for adipose cells treated with rilpivirine alone. Quercetin alone evoked a marked significant ($p < 0.001$) elevation in GSH activity. Adipose cells treated with a combination of rilpivirine and quercetin also showed a significant ($p < 0.01$) increase in the GSH activity as compared to control.

5.3.6 Immuno-fluorescence staining with adipogenic marker antibodies

This set of experiment was designed to demonstrate both the presence and the subcellular localization of crucial protein markers involved in the process of adipogenesis. Briefly, adipose cells were grown, treated with either rilpivirine or quercetin alone and in combination, fixed and stained directly in 96 well black-coloured plates using primary antibodies for specific adipogenic markers. goat anti-rabbit fluorochrome-conjugated secondary antibody, Alexa Fluor was used to stain subcellular proteins while DAPI was used to stain the nuclei.

6.3.6.1 Morphological and quantitative measurement of PPAR γ

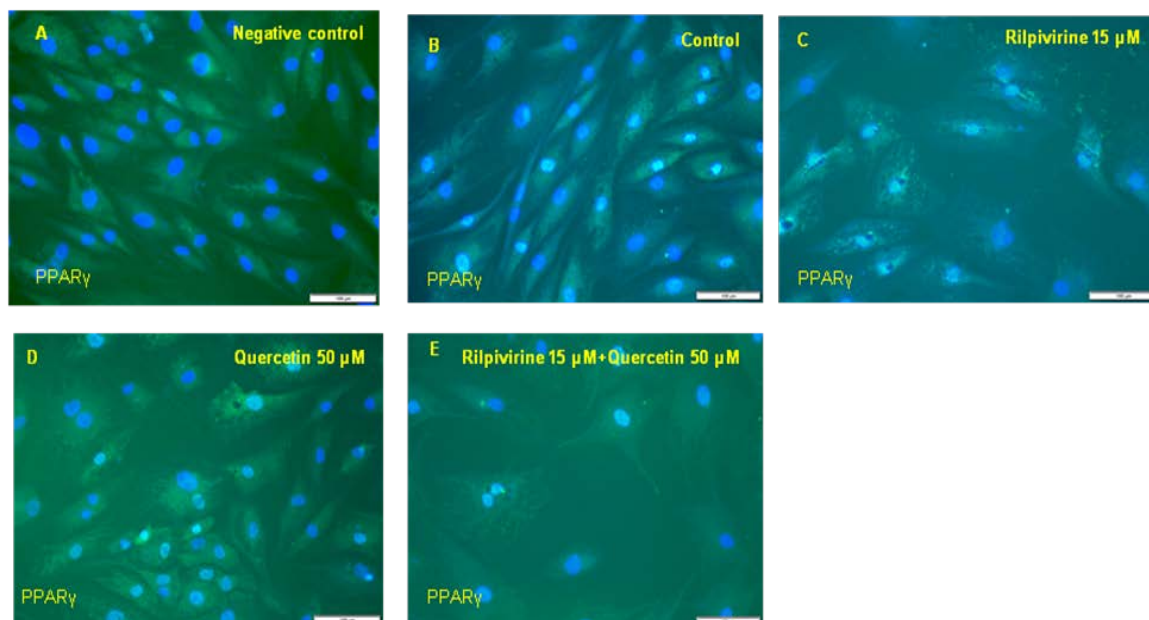


Figure 5.13: Images A-E showing the expression of PPAR γ adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. PPAR γ (green) was detected using PPAR γ primary antibody diluted at 1:100 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M.

- A. Negative control without the primary antibody.
- B. Differentiated adipose cells without any drug treatment (Control).
- C. Differentiated adipose cells treated with 15 μ M rilpivirine.
- D. Differentiated adipose cells treated with 50 μ M quercetin.

E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

Morphological data presented in figure 5.13 (A-E) show a reduced expression of PPAR γ for adipose cells treated with either rilpivirine or quercetin alone and in combination as compared to control. These images for PPAR expression were quantified and the results are shown in figure 6.14.

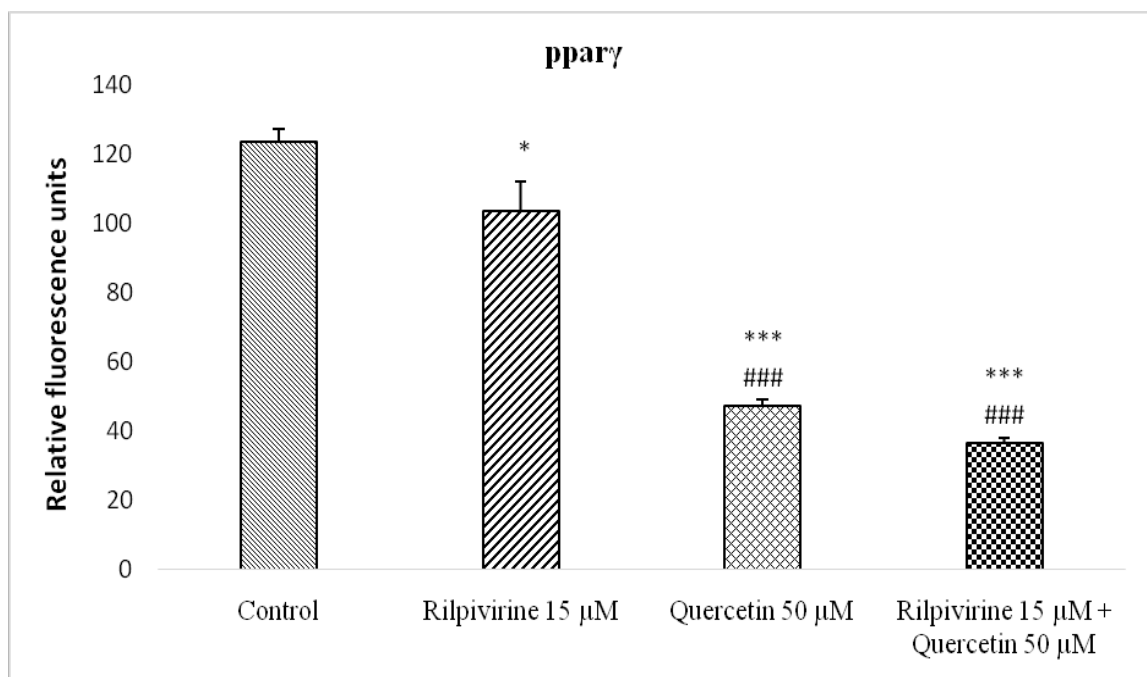


Figure 5.14: Bar charts showing the quantitative measurement of PPAR in human adipose cells treated with either rilpivirine or quercetin alone and in combination. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 μ M rilpivirine and 50 μ M quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. # represents significance difference within the treatment groups.

The data presented in figure 5.14 show significant decreases (p <0.05) in PPAR expression for adipose cells treated with rilpivirine as compared to control. Quercetin showed a statistically sharp reduction (p <0.001) in PPAR levels as compared to control as well as rilpivirine. Treatment

with a combination of rilpivirine and quercetin also showed a significant ($p<0.001$) decrease in PPAR expression as compared to control.

5.3.6.2 Morphological and quantitative measurement of CEBP

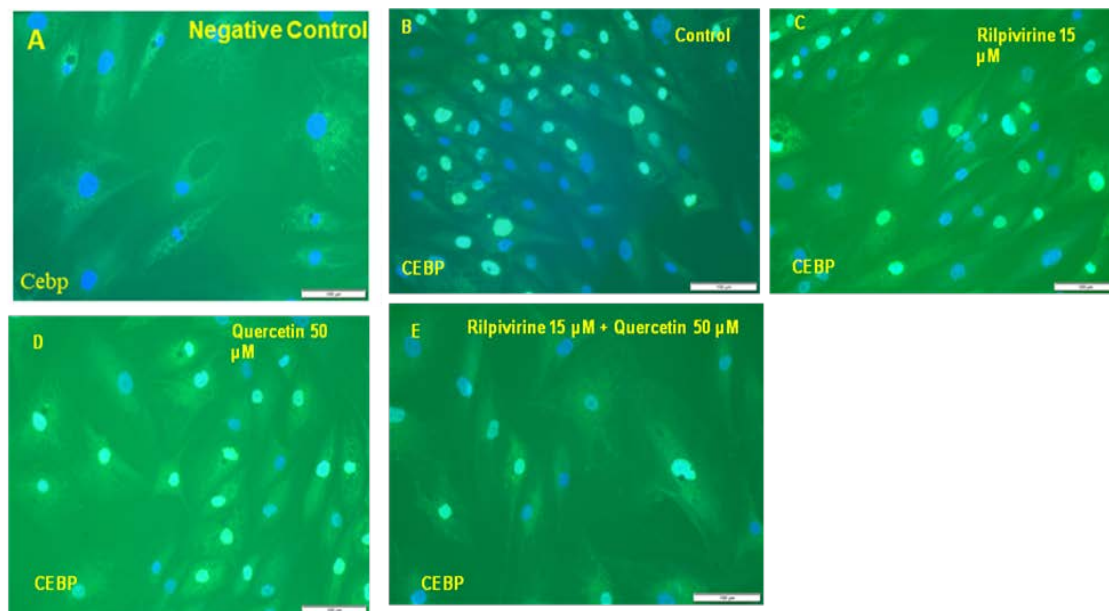


Figure 5.15: Images A-E showing expression of CEBP adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. CEBP (green) was detected using CEBP primary antibody diluted at 1:100 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M.

- A. Negative control without the primary antibody.
- B. Differentiated adipose cells without any drug treatment (Control).
- C. Differentiated adipose cells treated with 15 μ M rilpivirine.
- D. Differentiated adipose cells treated with 50 μ M quercetin.
- E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

The morphological data presented in figure 5.15 (Images A-E) show a reduced expression of CEBP adipogenic marker for adipose cells treated with either rilpivirine or quercetin alone and in

combination. These images for CEBP expression were quantified and the results are shown in figure 6.16.

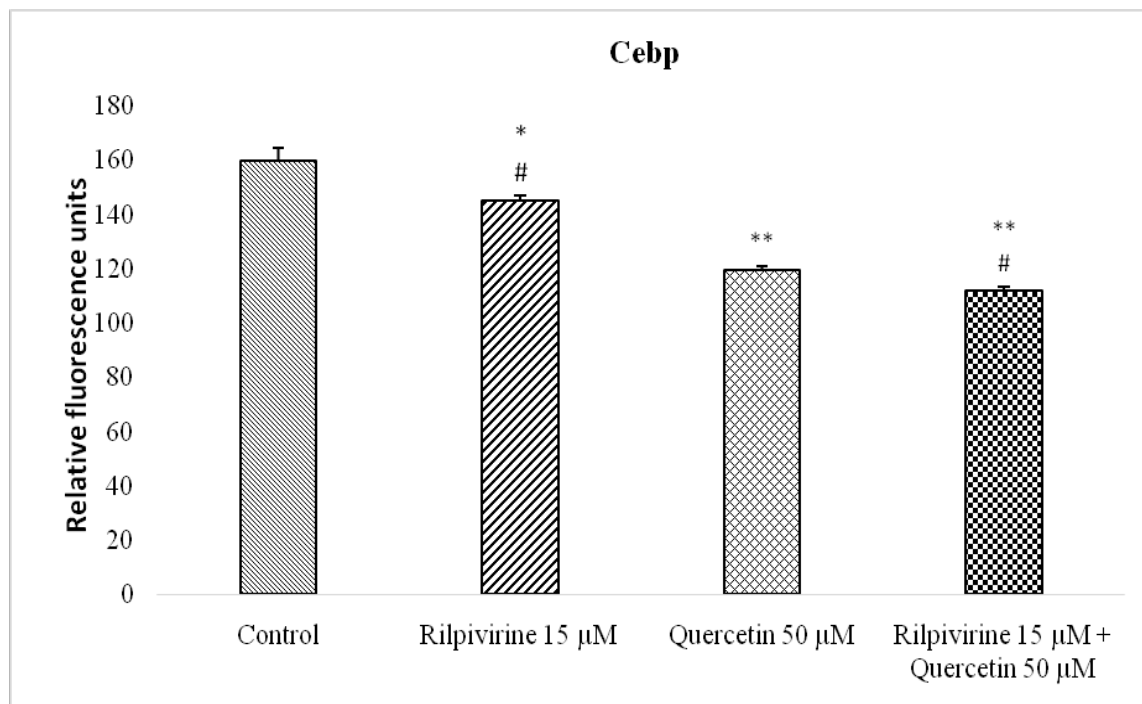


Figure 5.16: Bar graphs showing the quantitative measurement of CEBP in human adipose cells treated with either rilpivirine or quercetin alone and in combination. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The data presented in figure 5.16 show a statistically significant decrease ($p < 0.05$) in CEBP expression for the adipose cells treated with rilpivirine as compared to control. Treatment with quercetin alone or its combination with rilpivirine also showed significant reduction ($p < 0.01$) in CEBP expression levels as compared to control.

5.3.6.3 Morphological and quantitative measurement of Perilipin

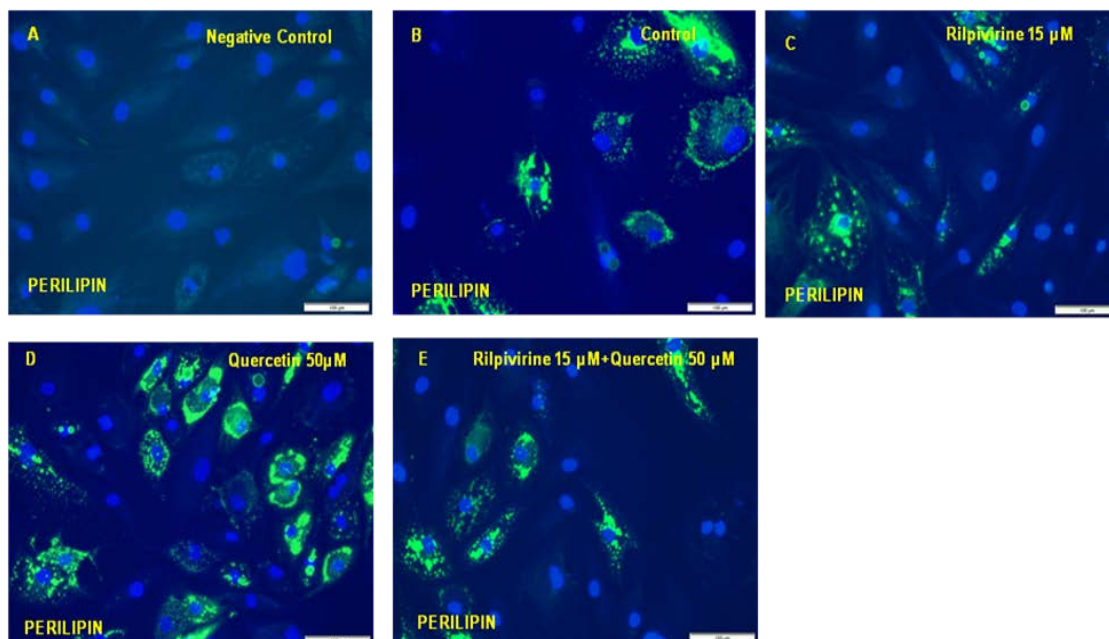


Figure 5.17: Images A-E showing expression of Perilipin adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Perilipin (green) was detected using perilipin primary antibody diluted at 1:200 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M.

- A. Negative control without the primary antibody.
- B. Differentiated adipose cells without any drug treatment (Control).
- C. Differentiated adipose cells treated with 15 μ M rilpivirine.
- D. Differentiated adipose cells treated with 50 μ M quercetin.
- E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

The data, presented in figure 5.17 (Images A-E), show a marked increase in the perilipin protein levels for adipose cells treated with either rilpivirine alone and in combination. These images for perilipin expression were quantified and the results are shown in figure 5.18.

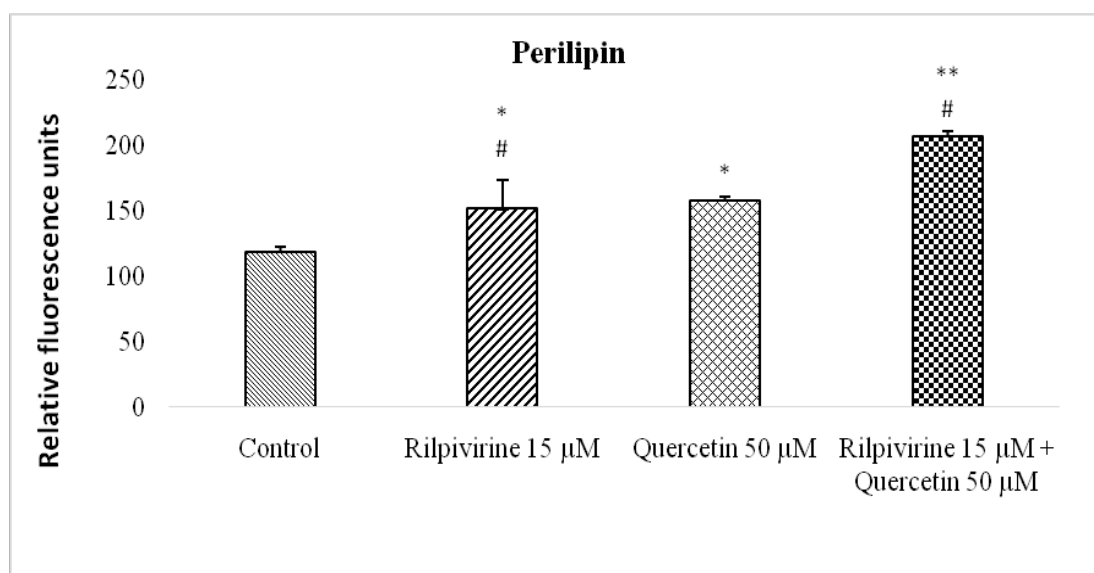


Figure 5.18: Bar charts showing the quantitative measurement of Perilipin in human adipose cells treated with either rilpivirine or quercetin alone and in combination. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine, 50 µM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The results presented in figure 5.18 show significant increases ($p < 0.05$) in perilipin expression for adipose cells treated with either rilpivirine or quercetin. Combination of rilpivirine and quercetin showed a dramatic and significant ($p < 0.01$) elevation in perilipin expression as compared to control.

5.3.6.4 Morphological and quantitative measurement of Fatty acid synthase

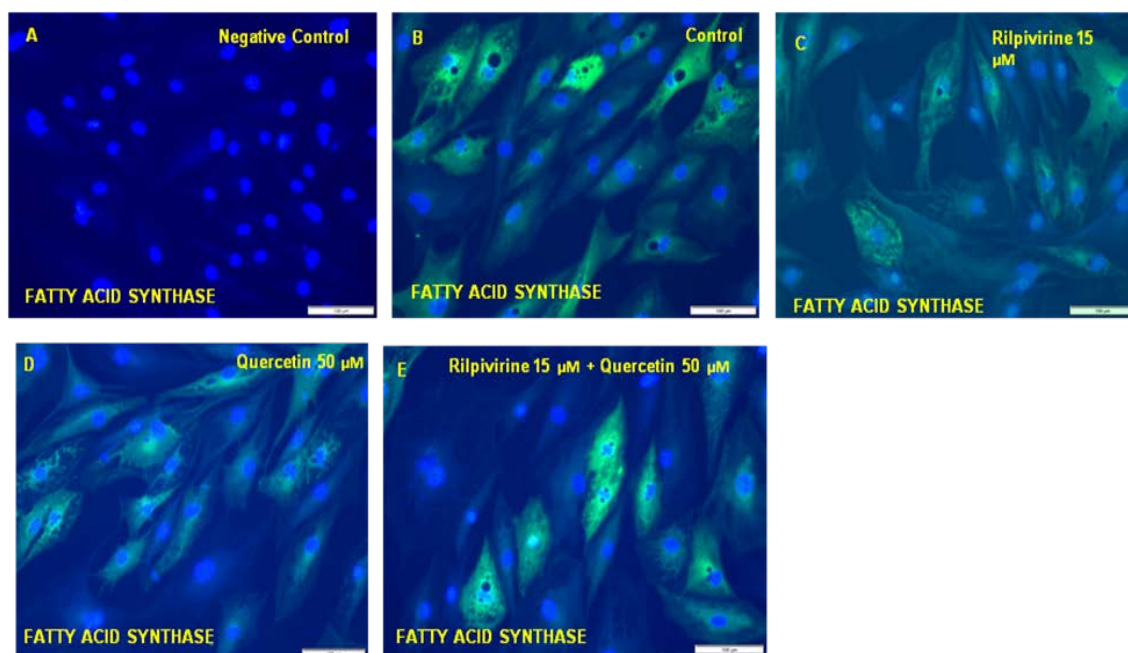


Figure 5.19: Images A-E showing the expression of fatty acid synthase adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Fatty acid synthase (green) was detected using FAS primary antibody diluted at 1:50 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M.

- A. Negative control without the primary antibody.
- B. Differentiated adipose cells without any drug treatment (Control).
- C. Differentiated adipose cells treated with 15 μ M rilpivirine.
- D. Differentiated adipose cells treated with 50 μ M quercetin.
- E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

The morphological data, presented in figure 5.19 (Images A-E), show a reduced expression of fatty acid synthase for adipose cells treated with either rilpivirine or quercetin alone and in combination. These images for fatty acid synthase expression were quantified and the results are shown in figure 5.20.

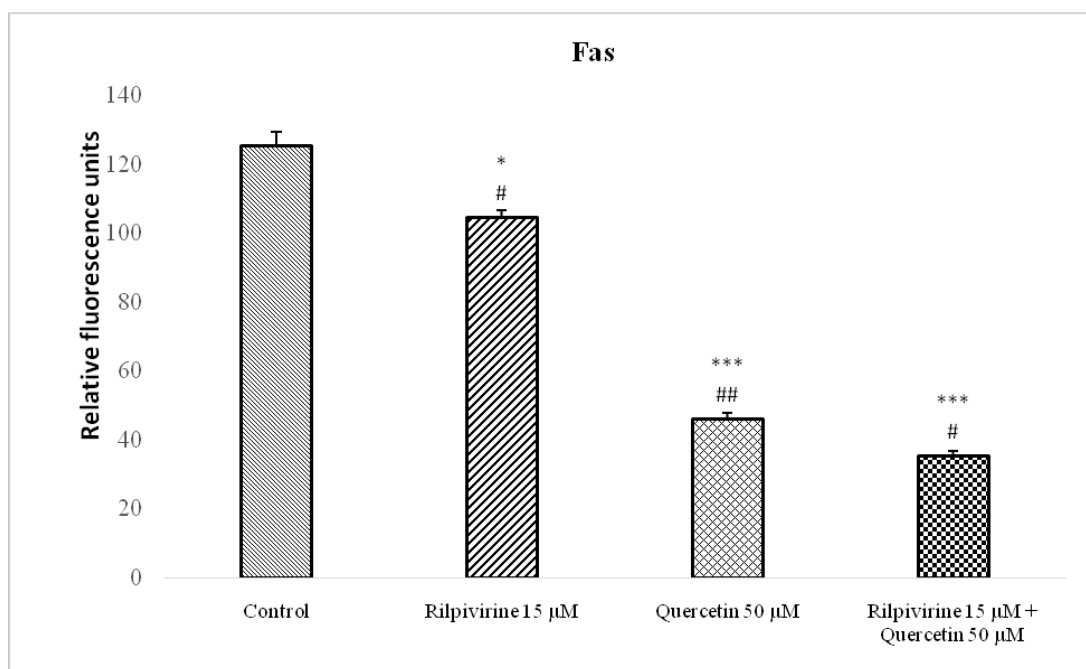


Figure 5.20: Bar charts showing the quantitative measurement of fatty acid synthase in human adipose cells treated with either rilpivirine or quercetin alone and in combination. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The data presented in figure 5.20 show a small but significant ($p < 0.05$) decrease in fatty acid synthase levels for adipose cells treated with rilpivirine. Significant reduction ($p < 0.001$) in FAS was observed for adipocytes treated with quercetin alone or its combination with rilpivirine.

5.3.6.5 Morphological and quantitative measurement of Fatty acid binding protein-4 (FABP4)

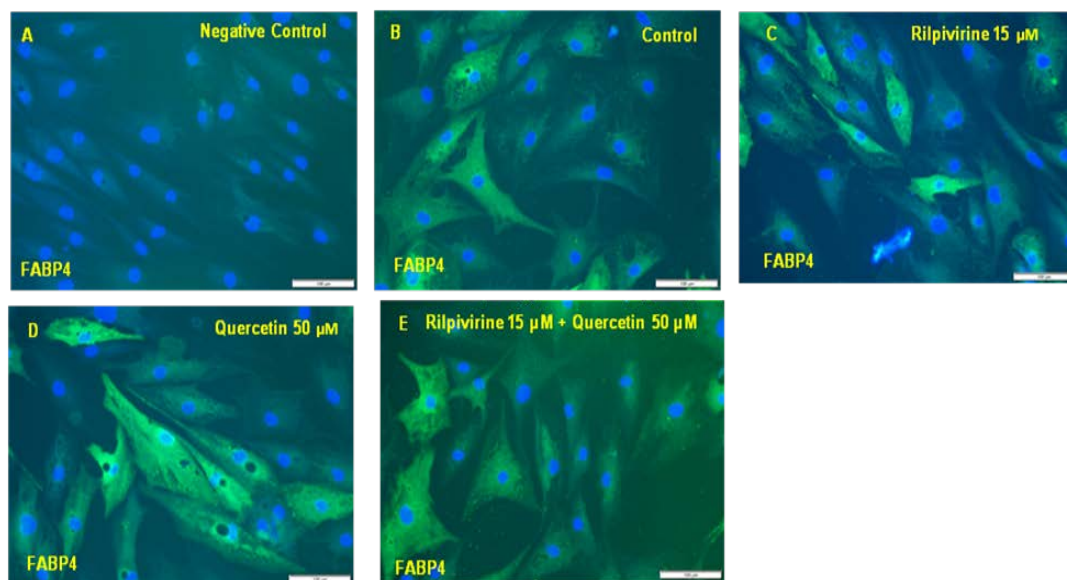


Figure 5.21: Images A-E showing the expression of FABP4 adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Fatty acid binding proteins (green) were detected using FABP4 primary antibody diluted at 1:200 concentrations. Nuclei (blue) were stained with DAPI. Cells were imaged at 20 X magnification with a scale bar of 100 μ M.

A. Negative control without the primary antibody.

B. Differentiated adipose cells without any drug treatment (Control).

C. Differentiated adipose cells treated with 15 μ M rilpivirine.

D. Differentiated adipose cells treated with 50 μ M quercetin.

E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

The morphological data, presented in figure 5.21 (Images A-E), show a reduced expression of FABP4 for adipose cells treated with either rilpivirine or quercetin alone and in combination. These images for FABP4 expression were quantified and the results are shown in figure 5.22.

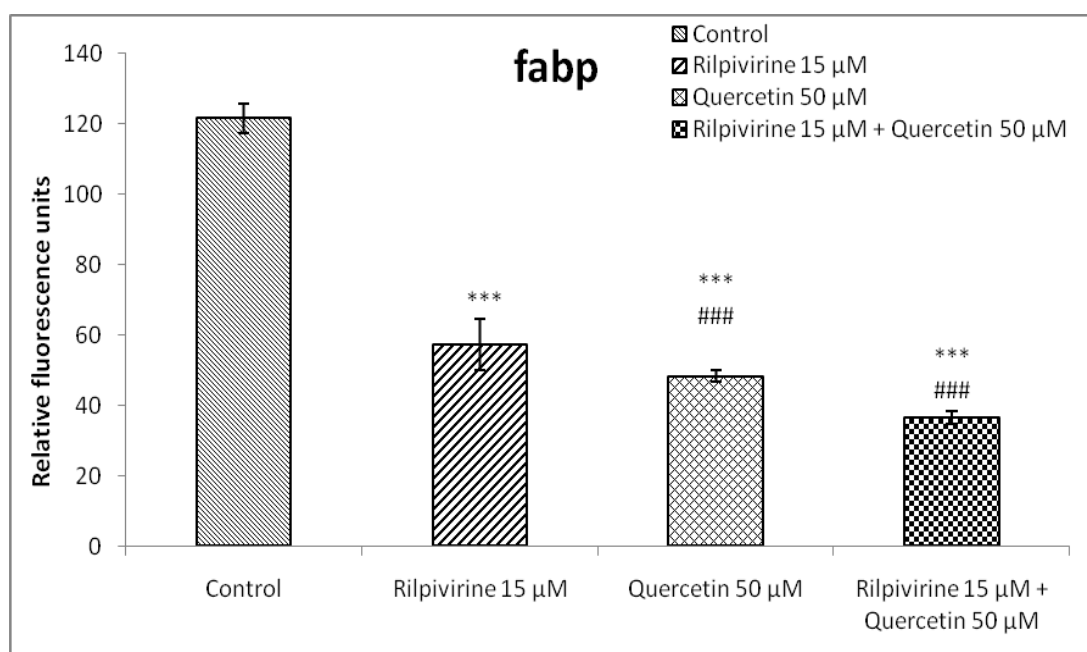


Figure 5.22: Bar charts showing the quantitative measurement of fatty acid binding proteins in human adipose cells treated with either rilpivirine or quercetin alone and in combination.

Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The data presented in figure 5.22 show significant ($p < 0.001$) differences for all treatment groups as compared to control. The results show that rilpivirine can significantly ($p < 0.001$) reduce the levels of FABP4. Treatment with quercetin alone and in combination with quercetin also showed a sharp and significant ($p < 0.001$) reduction in FABP4 expression.

5.3.6.6 Morphological and quantitative measurement of Acetyl coenzyme carboxylase (ACC)

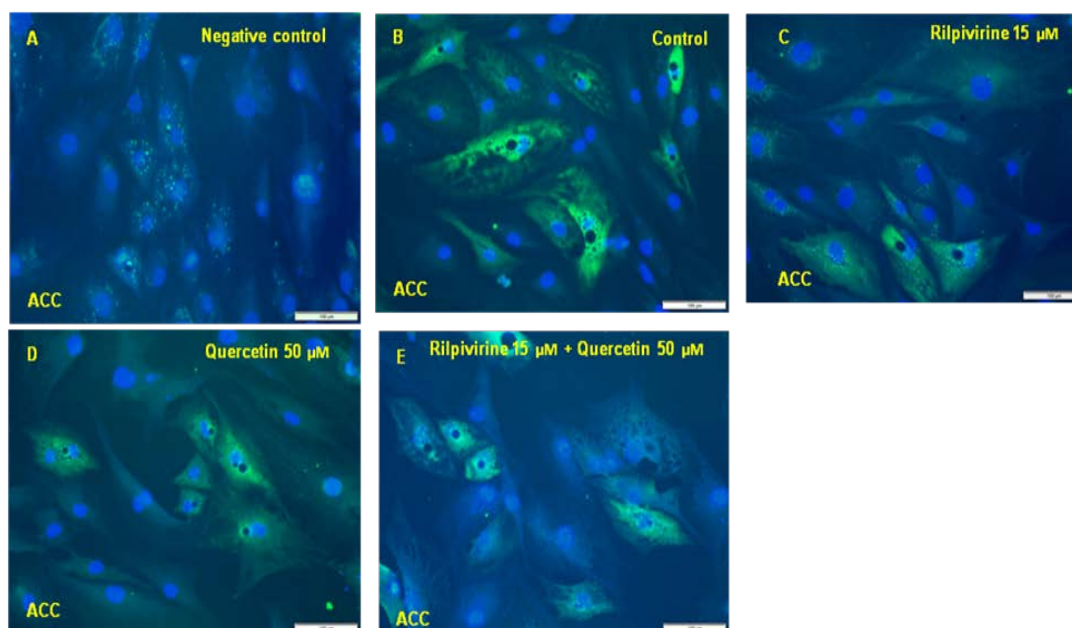


Figure 5.23: Images A-E showing the expression of Acetyl-CoA carboxylase adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. ACC (green) was detected using ACC primary antibody diluted at 1:200 concentrations. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M.

- A. Negative control without the primary antibody.
- B. Differentiated adipose cells without any drug treatment (Control).
- C. Differentiated adipose cells treated with 15 μ M rilpivirine.
- D. Differentiated adipose cells treated with 50 μ M quercetin.
- E. Differentiated adipose cells treated with a combination of 15 μ M rilpivirine and 50 μ M quercetin. These images are typical of 3 different experiments.

The morphological data, presented in figure 5.23 (Images A-E), show a reduced expression of ACC for adipose cells treated with either rilpivirine or quercetin alone and in combination. These images for ACC expression were quantified and the results are shown in figure 5.24.

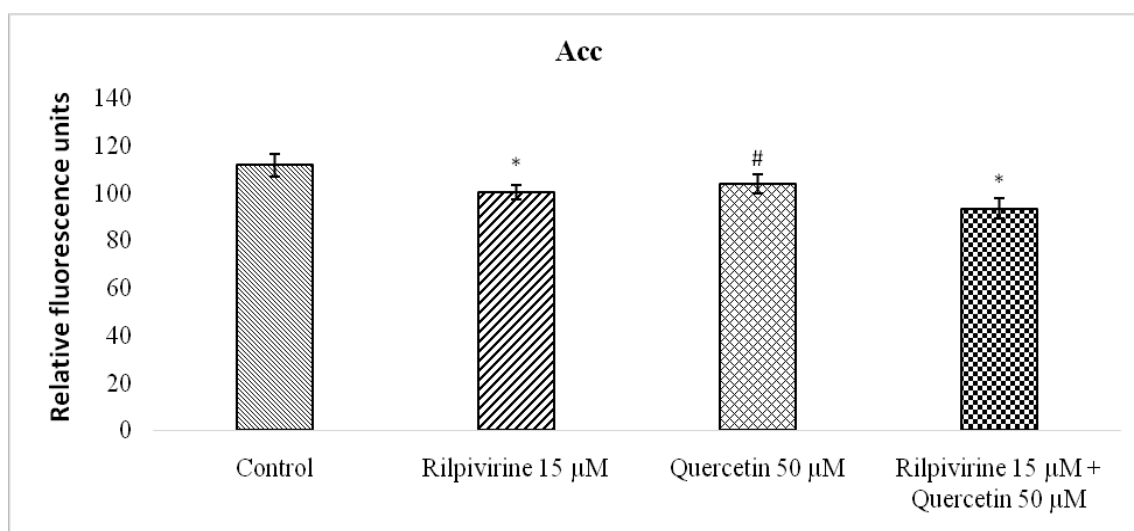


Figure 5.24: Bar charts showing the quantitative measurement of Acetyl-CoA carboxylase in human adipose cells treated with either rilpivirine or quercetin alone and in combination.

Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 µM rilpivirine and 50 µM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. # represents significance difference within the treatment groups.

The results presented in figure 5.24 show a small but significant decrease ($p < 0.05$) in the ACC expression for adipose cells treated with rilpivirine. Quercetin also reduced the ACC levels, but not significantly ($p < 0.05$) different as compared to control. A combination of quercetin and rilpivirine evoked a significant ($p < 0.05$) reduction in ACC levels compared to control.

5.3.7 Gene expression analysis using RT-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to determine the level of mRNA expression for adipocyte-specific genes and major adipogenic transcriptional factors (PPAR- γ , SREBP-1, C/EBP, LPL, aP2 and FAS) using specific primers and commercially available kits. Briefly, on day 8 after differentiation, total RNA was extracted from cultured cells and reverse transcribed. PCR analyses were then performed on cDNA preparation aliquots to detect mRNA expression. The amount of cDNA templates used for PCR was determined within

the linear range. A 10 μ L aliquot from each PCR reaction was electrophoresed in a 1.5 % agarose gel containing 0.2 μ g/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. The levels of adipogenic marker genes were expressed after normalization with the β -actin signal from the same sample.

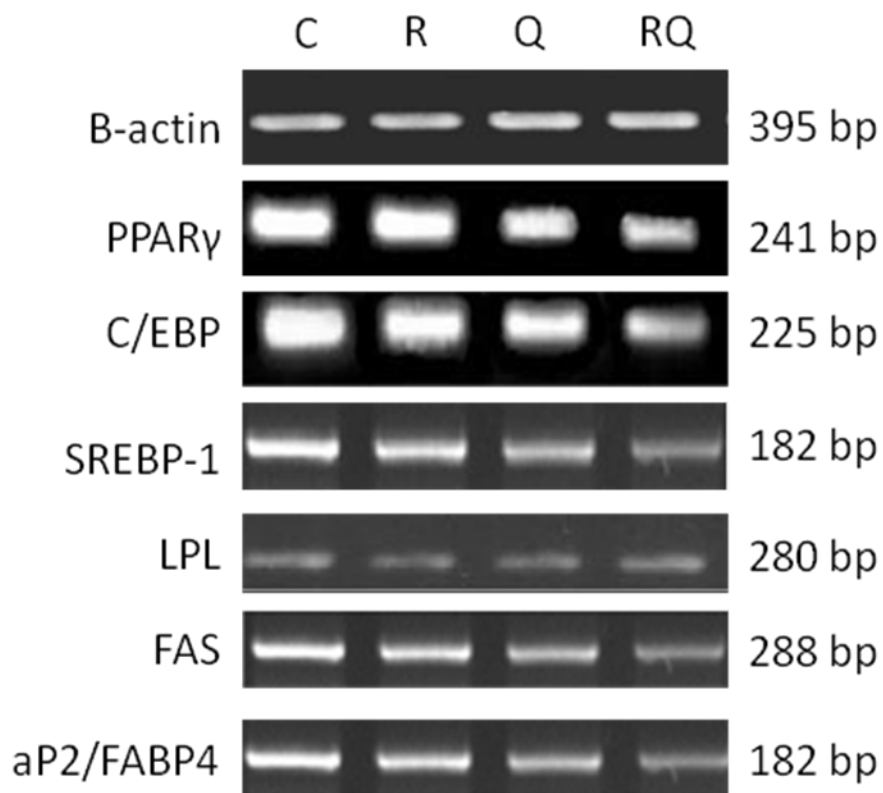


Figure 5.25: Original RT-PCR analysis of the mRNA expression of adipogenic markers. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (15 μ M rilpivirine, 50 μ M quercetin) individually and then in combination. After 8 days, the mRNA expression levels of PPAR γ , C/EBP α , SREBP-1, LPL, FABP4 and FAS were measured using RT-PCR.

Data from figure 5.25 show that the expression of adipogenic markers is markedly reduced for adipose cells treated with either rilpivirine or quercetin when compared to control. This reduction is more pronounced when both the drugs are combined.

5.4 Discussion

The introduction of highly active antiretroviral therapy (HAART) in the mid-1990s has dramatically decreased morbidity and mortality rates among HIV-infected patients (Detels et al., 1998; Palella et al., 1998). However, multiple morphologic and metabolic abnormalities have

been observed among HAART-treated patients with well-controlled HIV, including subcutaneous adipose tissue wasting (lipo-atrophy), central adipose tissue accumulation (lipo-hypertrophy), severe dyslipidemia and abnormalities of glucose metabolism. Previous data on anti-retroviral drug therapy have demonstrated serious metabolic consequences (FRAM, 2005). Although the new anti-retroviral drugs are not free of metabolic abnormalities, previous studies determining the effects on metabolic indices and body composition are limited. With this preview, the present study was therefore designed and conducted to determine any detrimental effects of rilpivirine on adipose cells, a newly developed HIV drug and its nutritional management using quercetin. Besides being an anti-inflammatory and anti-oxidative agent, quercetin has been shown to possess anti-viral properties as well (Mendonça-Murata et al., 2014).

The experiments in the present study were conducted in two series. In the first series of experiments, human subcutaneous adipose cells were treated with rilpivirine or quercetin alone. The second series of experiments were conducted to test the role of rilpivirine on the adipogenesis of adipose cells in combination with quercetin.

To measure the cyto-toxicity of cells, human subcutaneous adipose cells were treated with various drug concentrations of either rilpivirine or quercetin alone and in combination and viability was assessed on day 3 and 15 of culture. The results show that rilpivirine at lower concentrations (10 μ M and 15 μ M) and quercetin at 50 μ M did not alter the viability rate of the cells. However, at a concentration of 20 μ M, a slight and significant ($p < 0.05$) decrease in the cell viability was observed indicating its effect at higher doses. A combination of rilpivirine and quercetin at drug concentration of 20 μ M and 50 μ M respectively, also showed a significant ($p < 0.05$) decrease.

In the next series of experiments, human subcutaneous adipose cells were treated with various concentrations of rilpivirine or quercetin alone and then in combination with quercetin for a period of 15 days to determine triglyceride accumulation. Previous clinical trials conducted to measure the blood triglycerides, total cholesterol, low and high density lipoprotein in patients treated with rilpivirine showed substantial increases in these parameters compared to other NNRTIs belonging to the same class of antiretroviral drugs (Cohen et al., 2011; Molina et al., 2011; Nelson et al., 2013). These findings were confirmed by further studies (Wilkin et al., 2012). The results from the present study show substantial reduction in the accumulation of triglyceride levels for adipose cells treated with either rilpivirine or quercetin alone and in combination, as evidenced by morphological pictures and quantitative measurement of

triglycerides on the 5th, 8th, 11th and 15th day of the differentiation process. As compared to control, an altered lipid accumulation was observed in a dose- and time- dependent manner. Higher dose concentration of rilpivirine (20 μ M) alone or in combination with quercetin severely inhibited triglyceride accumulation during adipocyte differentiation. Based on these results, a median drug concentration of 15 μ M of rilpivirine and differentiation for a period of 8 days was chosen for further experiments.

One of the insights that changed our appreciation of the role of adipocytes over recent years has been the discovery of their hormonal role in the regulation of metabolism, energy intake and fat storage. It is currently known to secrete a large number of proteins termed adipokines that act in an autocrine, paracrine or endocrine fashion to control various metabolic functions. Alterations in inflammatory status of adipose tissue with obesity feed a growing recognition that obesity represents a state of chronic low-level inflammation. In the present study, human subcutaneous adipose cells were treated with various concentrations of either rilpivirine or quercetin individually and in combination with quercetin for a period of 8 days. Rilpivirine showed a marked reduction in adiponectin levels. Levels of other inflammatory markers such as resistin and IL-8 were increased as compared to the untreated cells. No significant changes in leptin were observed on treatment of adipose cells with rilpivirine alone and its levels were almost comparable to control. However, significant reduction in inflammatory markers was observed for adipose cells treated with quercetin alone, thereby, revealing its anti-inflammatory properties. Combination of the two drugs together, markedly reduced the inflammatory markers.

Antiretroviral therapy may often result in biochemical and physiological changes e.g. oxidative damage resulting from the presence of free radicals or the absence of antioxidants. So far, sufficient evidence to substantiate the role of free radicals in mediating oxidative injury is not present. Data from a series of previously conducted studies have reported an increase in oxidative stress, in addition to the persistent redox imbalance associated with HIV-1 infection manifested by an increase in oxidants and a decrease in antioxidant serum levels (Masiá et al., 2007; Mandas et al., 2009; Deavall et al., 2012). Specifically, a study done in 84 HIV infected patients during a 6-month period of ART demonstrated a significant increase in serum peroxidation potential, total hydro-peroxide and advanced oxidation protein product levels as well as a decrease in glutathione level, compared to their levels before the treatment and to healthy controls (Gil et al., 2011). An aggravation of the oxidative stress by certain ART regimens in the

form of a significant decrease in the levels of GSH (sulfhydryl group) has also been registered (Ngondi et al., 2006). In order to reveal the oxidative status of the adipose cells treated with a concentration of 15 μ M of rilpivirine individually and then in combination with 50 μ M of quercetin, biomarkers of oxidative stress were measured quantitatively using commercially available kits. The generated oxidative profile in this study revealed a significant decrease in the levels of anti-oxidant enzymes like superoxide dismutase, catalase and glutathione for adipose cells treated with rilpivirine. The results also show a significant increase in the nitric oxide levels indicating its role in mitochondrial dysfunction. This reduction in the anti-oxidant species may possibly be attributed to the production of more oxidized metabolites deriving from the interaction between ROS and infected cell biomolecules (de la Asunción et al., 1998; Kumar et al., 1999; Hulgán et al., 2003; Lewis, 2003; Cossarizza et al., 2004; Day et al., Lewis, 2004). It may further be explained and supported by several biochemical mechanisms, such as mitochondrial interference following treatment with ART (de la Asunción et al., 1998; Cossarizza et al., 2004; Day et al., 2004) and activation of the P450 hepatic system (Kumar et al., 1999). Either insufficient intake or mal-absorption of nutrients—common in HIV patients—may further worsen these conditions (Tang et al., 2005; Drain et al., 2007) .

In hope to lessen the side effects of antiretroviral therapy like lipo-dystrophy syndrome, rilpivirine at a concentration of 15 μ M was supplemented with 50 μ M of an anti-oxidant, quercetin. The results show that quercetin supplementation significantly ameliorated rilpivirine-induced oxidative stress. It was able to prevent injury caused by free radicals by scavenging of ROS, activate antioxidant enzymes and mitigate the oxidative stress caused by nitric oxide. In previously conducted preclinical *in vitro* studies, quercetin was shown to significantly reduce levels of inflammatory mediators such as NO synthase in human hepatocyte-derived cell line (García-Mediavilla et al., 2007). Nitric oxide reacts with free radicals, thereby producing high damaging peroxynitrite. Peroxynitrite can directly oxidize low density lipoproteins resulting in irreversible damage to cell membranes. Quercetin causes scavenging of free radicals; therefore can no longer react with nitric oxide, resulting in less damage (Shutenko et al., 1999). The overall increase in the levels of anti-oxidant enzymes when supplemented with quercetin, may be attributed to its much reported anti-oxidative and direct radical scavenging properties. Quercetin, thus seems to be a powerful flavonoid for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage (Groot, 1994; Grace, 1994).

The process of adipogenesis involves the differentiation of pre-adipocytes into mature adipocytes and it plays a key role in the expansion of adipose tissue mass and subsequent metabolic syndrome. This process is regulated by an elaborate network of transcription factors that coordinate expression of hundreds of proteins responsible for establishing the mature fat-cell phenotype. At the center of this network are the two principal adipogenic factors, PPAR γ and C/EBP α , which oversee the entire terminal differentiation process. PPAR γ in particular is considered as the master regulator of adipogenesis; without it, precursor cells are incapable of expressing any known aspect of the adipocyte phenotype (Rosen et al., 2000). On the other hand, cells deficient in C/EBP α are capable of adipocyte differentiation; however, these C/EBP α -deficient cells are insulin resistant (El-jack et al., 1999; Wu et al., 1999). Both PPAR γ and C/EBP α are greatly induced during adipogenesis, and they are necessary for the expression of many adipogenic genes such as perilipin, ACC, fatty acid synthase (FAS), adipocyte fatty acid-binding protein (aP2) (Wu et al., 1999; Farmer, 2005; White et al., 2010), and lipoprotein lipase (LPL) (Bae et al., 2014). As described earlier, secondary effects, including disturbances in lipid metabolism and ultimately, in adipose tissue distribution and function, are common concerns associated with antiretroviral treatments tested so far. It may, therefore, be important to study the expression of some of these markers involved in the process of adipogenesis. This study provides the first assessment of the effect of rilpivirine on the protein and gene expression of important markers involved in the process of adipogenesis. In this study, when pre-adipocytes were differentiated in the presence of 15 μ M of rilpivirine, the expression of PPAR γ , C/EBP α and their related genes and markers were highly altered, indicating an essential role for these transcription factors in the regulation of adipogenesis.

In parallel with the suppression of PPAR γ and C/EBP α expression by rilpivirine, the expression of SREBP-1 and its target genes like FAS, aP2 and LPL were all significantly decreased by rilpivirine treatment displaying the lipoatrophic effects of rilpivirine. The results demonstrate that rilpivirine alters adipogenesis through the down-regulation of adipogenic transcriptional factors and their target genes.

As adipogenesis plays a key role in obesity, the marked inhibition of adipogenesis by rilpivirine treatment and its subsequent amelioration by an herbal supplement may provide a significant clue as to the potential mechanisms by which flavonoid supplementation alter the adverse metabolic effects induced by antiretroviral drugs. The results from this study, show that quercetin treatment at a dosage concentration of 50 μ M suppressed the expression of PPAR γ ,

C/EBP α , SREBP-1 and their target genes. Co-administration of rilpivirine and quercetin further down regulated the expression of these markers.

In summary, this study reflects the lipotrophic effects of rilpivirine at high dosage concentrations (15 μ M) via inhibition of triglyceride accumulation and suppression of crucial markers of adipogenesis. It elucidates an increase in the inflammatory markers and suppresses the anti-oxidant enzymes. The study also, for the first time, reports co-administration of rilpivirine and quercetin. The results demonstrate the potential abilities of quercetin to attenuate oxidative damage and decrease inflammatory markers. However, it failed to overcome the lipotrophic affects of rilpivirine. Therefore, the study warrants further mechanistic and clinical research studies to develop quercetin as a proven natural and effective agent for the ART-induced prevention or treatment of metabolic disturbances. Knowledge of the potential interactions between ARVs and plant-derived products is very limited and scanty. More research needs to be done to identify potential harmful interactions. The information generated needs to be documented and made available to traditional healers, health practitioners and also to the HIV-infected individuals so that they can understand the impact that those interactions can have on the development of drug resistance and treatment failure.

Chapter 6

Effect of 17 β -estradiol and quercetin on human subcutaneous adipose cells *in vitro*

6.1 Introduction

It is well established that adipose tissue is a dynamic structure that is involved in the regulation of glucose and lipid metabolism, energy homeostasis and inflammation. Functional failure or dysfunction of adipose tissue may result in primary defects of obesity and may link excessive fat accumulation to several health problems including increased risk of insulin resistance, type 2 diabetes, fatty liver disease, hypertension, dyslipidemia, atherosclerosis, dementia, and certain forms of cancer (Blüher, 2013). The female sex hormone, estrogen, has been largely demonstrated to regulate adipose development (Björntorp, 1997; Schomberg et al., 1999; Heine et al., 2000; Clegg et al., 2006). It is known to promote, maintain, and control the typical distribution of body fat and adipose tissue metabolism and improve systemic glucose homeostasis in both males and females (Kim et al., 2014).

Estrogens are a family of compounds that include 17 alpha-estradiol (17α -E2) as well as 17 beta-estradiol (17β -E2). 17β -E2 is the main circulating and biologically most active form of estrogen (Mauvais-Jarvis et al., 2013). Although clinical and experimental evidences report estradiol as a major regulator of adiposity and regional distributor of fat (Gavin et al., 2013), its exact role on the inflammatory and oxidative status during adipocyte differentiation is currently still debatable. The underlying mechanisms linking estrogenic regulation in adipose tissue and systemic fatty acid metabolism has not yet been fully elucidated and is mainly reported to include interactions of estrogen receptor signaling events involving lipolytic and/or lipogenic enzyme activity, free fatty acid metabolism and adipocytokine production. An understanding of the effects of estrogen replacement on adipose tissue biology and metabolism is therefore important in determining the risk of developing obesity-related metabolic disorders in patients undergoing treatment for sex hormone deficiency.

Several plant compounds have been reported to possess estrogenic properties (Badger et al., 2002; Bhathena et al., 2002; Salleh et al., 2013). Quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one], the most widely distributed plant flavonoid is one of the major constituents of the human diet and is abundantly present in the outer skin of onions, apples, and berries (Peluso, 2006; Boots et al., 2008). This compound possesses a broad range of biological activities, including anti-inflammatory, anti-pathogenic, anti-oxidant, and immune-modulatory (Russo et al., 2012). Due to its ability to bind to the estrogen receptor (ER), quercetin has been categorized as a phyto-estrogen (Kuiper et al., 1996; Muthian et al., 2004). Apart from ER, quercetin was also reported to bind to the type II estrogen-binding site

(Caltagirone et al., 1997). Despite its reported estrogen-like activities, the effect of quercetin on uterine morphology and proliferation is currently unknown.

Aims: Therefore, the aims of the present study were to assess the effects of estradiol and quercetin on adipogenic markers, inflammatory markers and intracellular redox processes which may be involved in the pathogenesis of adiposity.

6.2 Materials and methods

As described in chapter 2.

6.3 Results

6.3.1 Measurement of cytotoxicity using lactate dehydrogenase assay

The human subcutaneous pre-adipocytes were seeded at a density of 2×10^4 cells per well in 24-well plates and cultured until 90 % confluence in culture medium. The cells were then treated with various concentrations of rilpivirine or quercetin either alone and in combination for a period of 15 days. As an indicator of cytotoxicity, the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the culture supernatant of the control as well as treated differentiating adipose cells on day 3 and day 15 of culture. An optimized LDH test was used to quantify LDH activity in the medium of the treated differentiating adipose cells. The percentage of viable cells was calculated by defining the cell viability without treatment as 100 %.

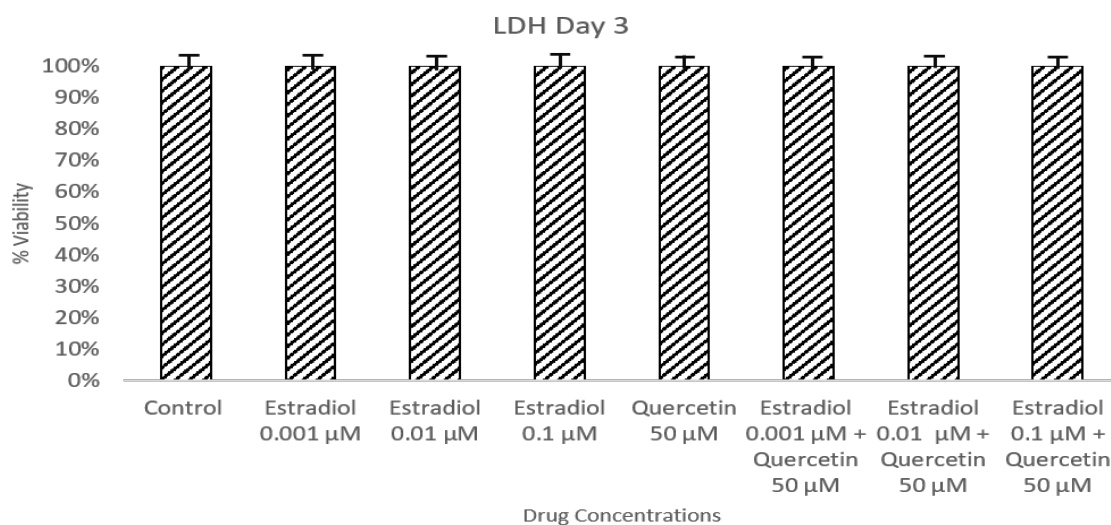


Figure 6.1: Bar graph showing the effect of either estradiol or quercetin either alone or in combination on the cell viability as measured by LDH on day 3 of culture. Human

subcutaneous adipose cells were treated with various concentrations of indicated drugs either alone and in combination. Cytotoxicity was expressed as the percentage of LDH activity in the medium as compared to control on day 3 of incubation. Results represent mean \pm SEM of 3 parallel cell culture experiments.

The results presented in figure 6.1 show no significant ($P>0.05$) changes in the viability of cells when treated with various concentrations of either estradiol or quercetin alone and in combination for day 3 of culture.

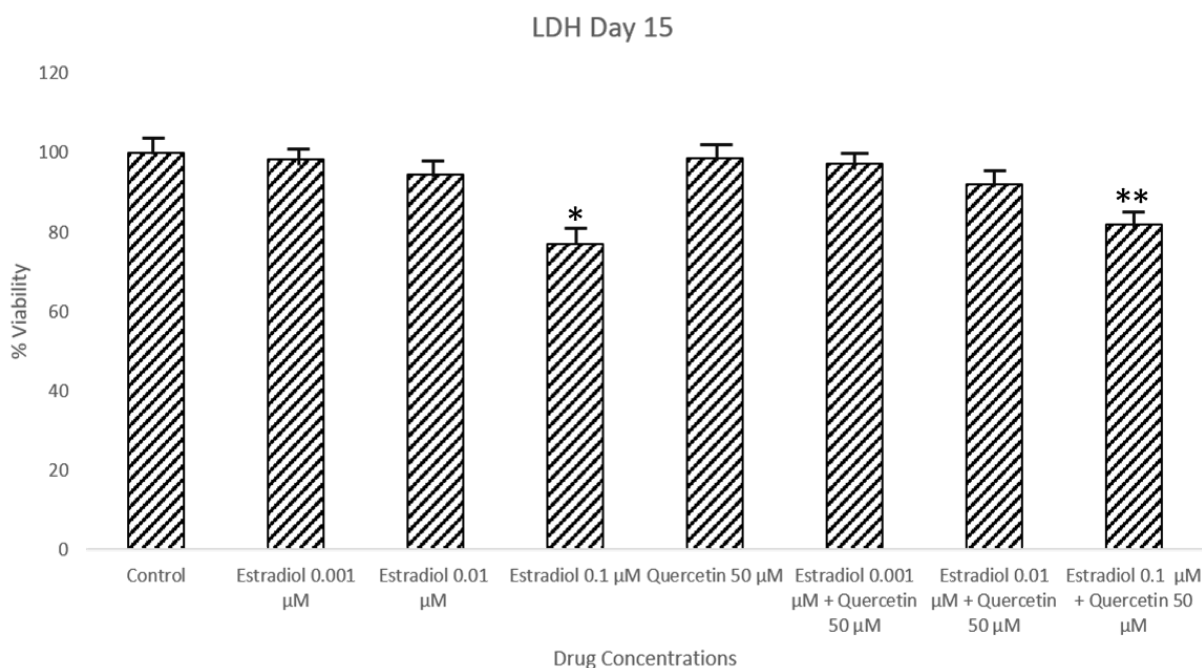
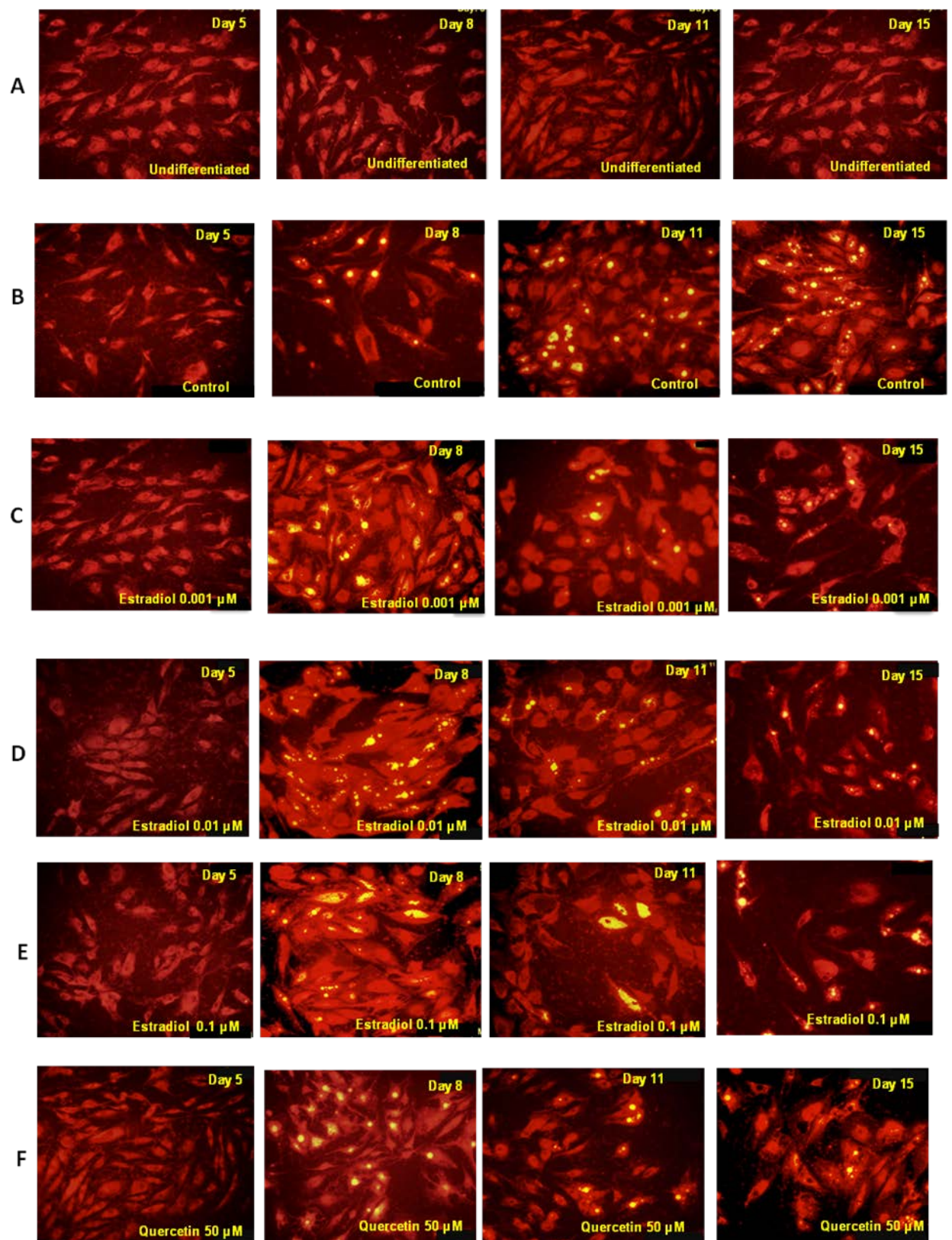


Figure 6.2: Bar graph showing the effect of either estradiol or quercetin either alone or in combination on the cell viability as measured by LDH on day 15 of culture. Human subcutaneous adipose cells were treated with various concentrations of indicated drugs either alone and in combination. Cytotoxicity was expressed as the percentage of LDH activity in the medium as compared to control on day 15 of incubation. Results represent mean \pm SEM of 3 parallel cell culture experiments. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared to the control.

The results presented in figure 6.2 show that estradiol at lower concentrations (0.001 μ M and 0.01 μ M) and quercetin at 50 μ M did not affect the viability rate of the cells. However, at a concentration of 0.1 μ M of estradiol either alone or in combination with 50 μ M of quercetin, a significant decrease in cell viability (cell death) ($p<0.05$ and $p<0.01$) respectively, was observed compared to non-treated cells.

6.3.2 Morphological examination of adipose cells using adipoRed staining



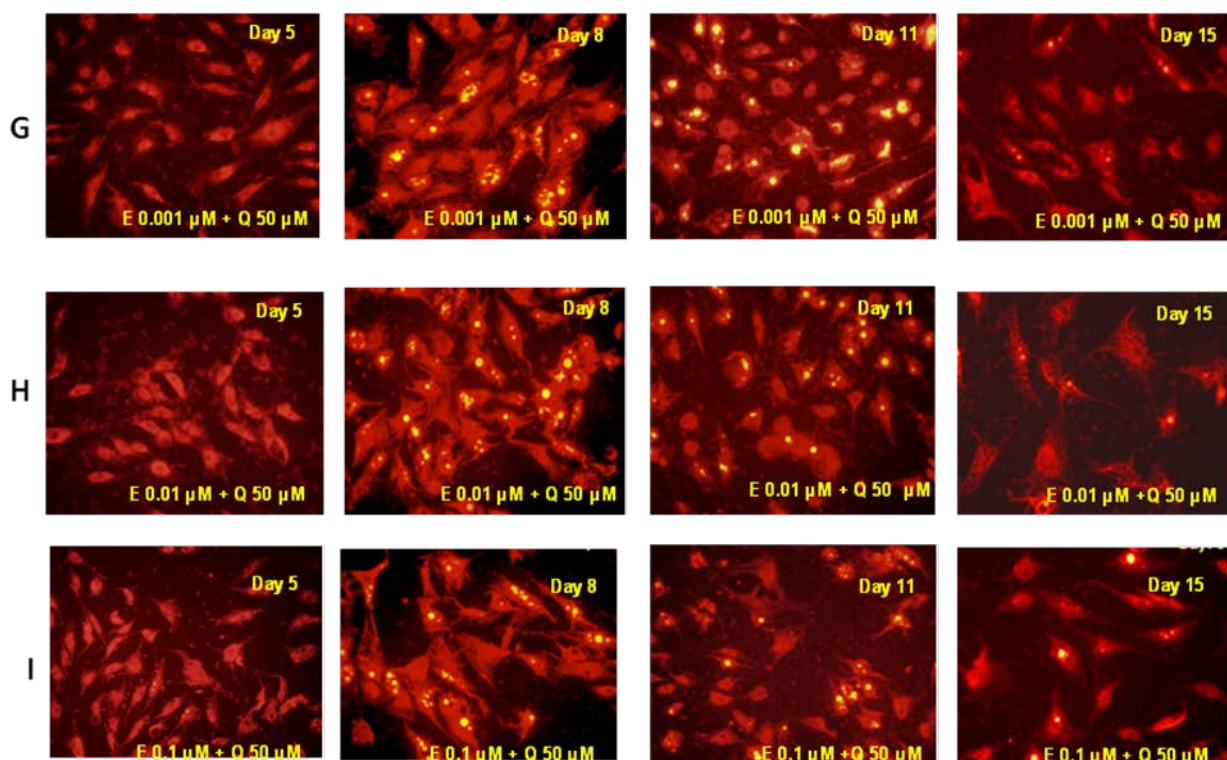


Figure 6.3: Photographs (36 in all) showing representative time course images (A-I) of triglyceride accumulation indicated by lipid formation for human adipose cells in culture. Human subcutaneous pre-adipocytes were differentiated and treated with different concentrations of either 17 β -estradiol (E2) or quercetin individually and then in combination. Images were taken for the 5th, 8th, 11th and 15th day of cells in culture at 20x magnification using a fluorescence microscope. Staining was done using adipored to visualize lipid content. Images are typical of 3 such parallel experiments.

- A. Undifferentiated and untreated (no drugs) adipose cells.
- B. Differentiated and untreated (no drugs) adipose cells.
- C. Adipocytes differentiated and treated with 0.001 μ M of estradiol.
- D. Adipocytes differentiated and treated with 0.01 μ M of estradiol.
- E. Adipocytes differentiated and treated with 0.1 μ M of estradiol.
- F. Adipocytes differentiated and treated with 50 μ M of quercetin.
- G. Adipocytes differentiated and treated with a combination of 0.001 μ M estradiol and 50 μ M quercetin.

H. Adipocytes differentiated and treated with a combination of 0.1 μ M estradiol and 50 μ M quercetin.

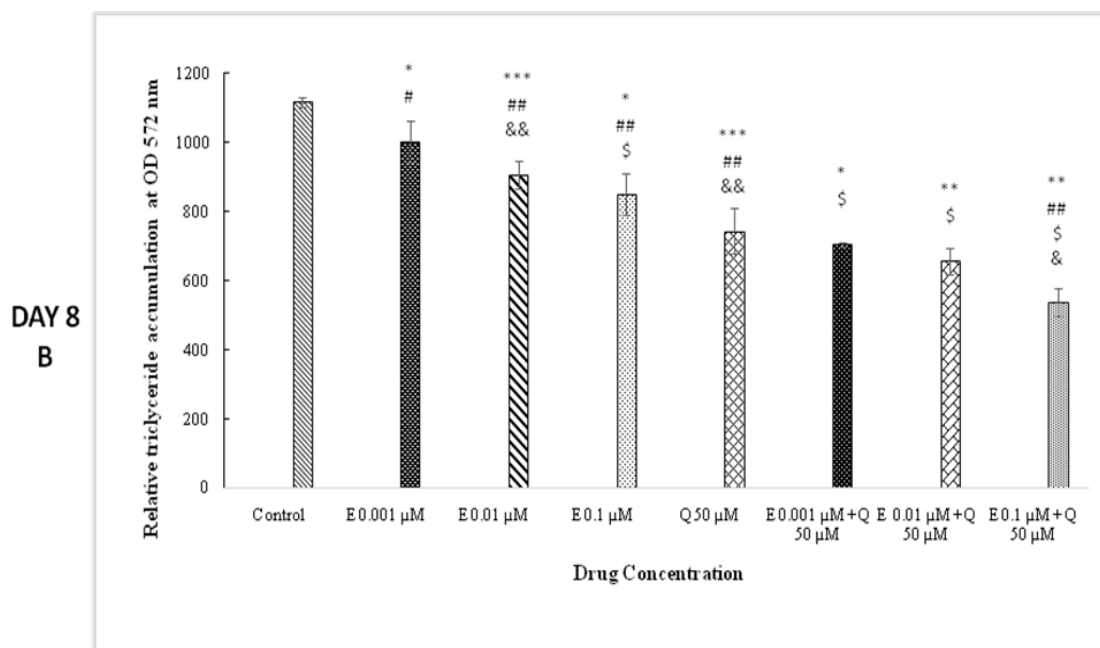
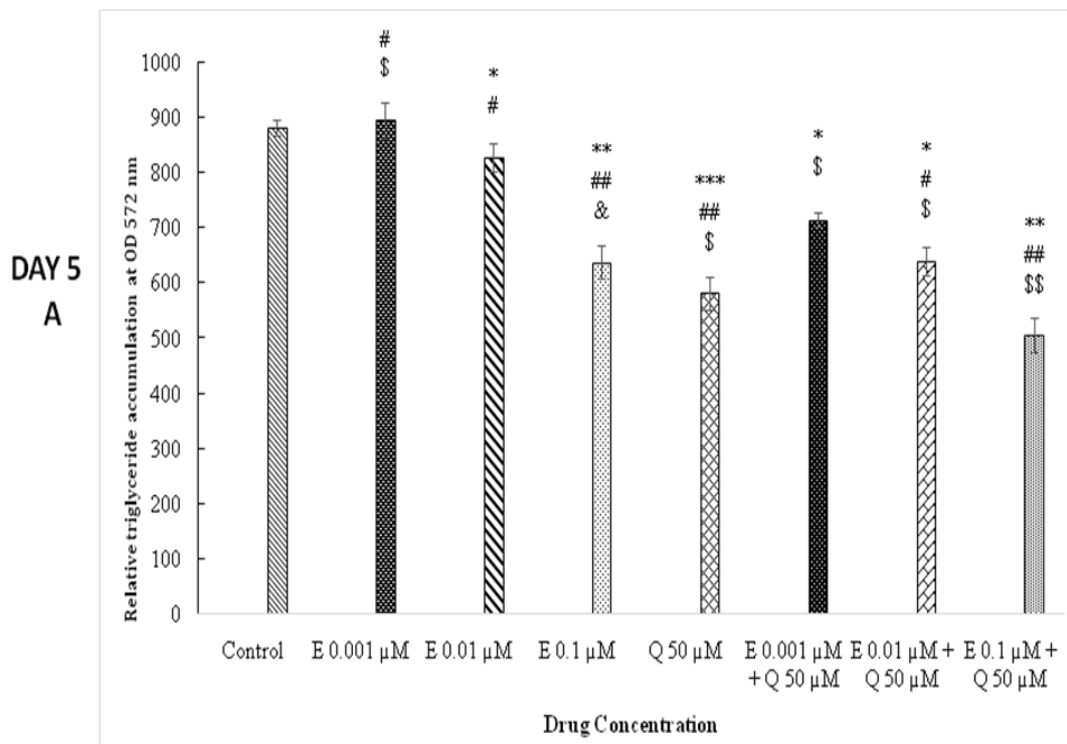
I. Adipocytes differentiated and treated with a combination of 0.1 μ M estradiol and 50 μ M quercetin.

Morphological data presented in figure 6.3 show the accumulation of triglyceride in pre-adipocytes cultured and differentiated in the presence/absence of treatment drugs either alone or in combination. Image A represents undifferentiated and untreated pre-adipocyte cells in culture. The data show that the undifferentiated pre-adipocytes appear elongated, fibroblast-like and spindle shaped and showed no lipid accumulation. Image B represents pre-adipocyte cells differentiated for a period of 15 days without any drug treatment (Control). The process of adipogenesis progressed in a time-dependent manner (5-15 days) as evidenced by the increasing lipid droplets (yellow colour). On day 5, almost none or very little amount of lipid droplets was observed. However, by day 8 the cells began to attain a round shape in structure and became laden with triglycerides and accumulated substantial lipid content. These lipid vacuoles were observed to accumulate more triglycerides and grow more in size and number on day 11 and 15 in culture. Images C, D and E represent pre-adipocyte cells treated with different concentrations of 0.001 μ M, 0.01 μ M and 0.1 μ M of estradiol respectively. It was observed that estradiol inhibited the accumulation of triglycerides in both dose- and time-dependent manner. The formation of lipid droplets (yellow colour) decreased with increasing doses and time in culture. Image F represents pre-adipocytes treated with 50 μ M of quercetin. Pre-adipocytes treated with quercetin were shown to inhibit triglyceride accumulation with increasing time in culture. The number of lipid droplets decreased from day 8 until day 15 in culture. Images G, H and I represent pre-adipocyte cells treated with a combination of 0.001 μ M, 0.01 μ M, 0.1 μ M concentrations of estradiol and 50 μ M of quercetin, respectively. Adipose cells treated with these combinations of drugs showed further attenuation in lipid formation in a time- and dose-dependent manner. At day 8 of differentiation the adipocyte cells accumulated a substantial amount of triglyceride which subsequently diminished gradually until day 15.

6.3.3 Quantitative measurement of triglycerides

Triglycerides accumulated over the differentiation period of 15 days were measured quantitatively by staining the cells with adipored reagent and the fluorescence was measured at

OD value of 572 nm. The data are presented in figure 6.4 (Images A-D for days 5, 8, 11 and 15, respectively).



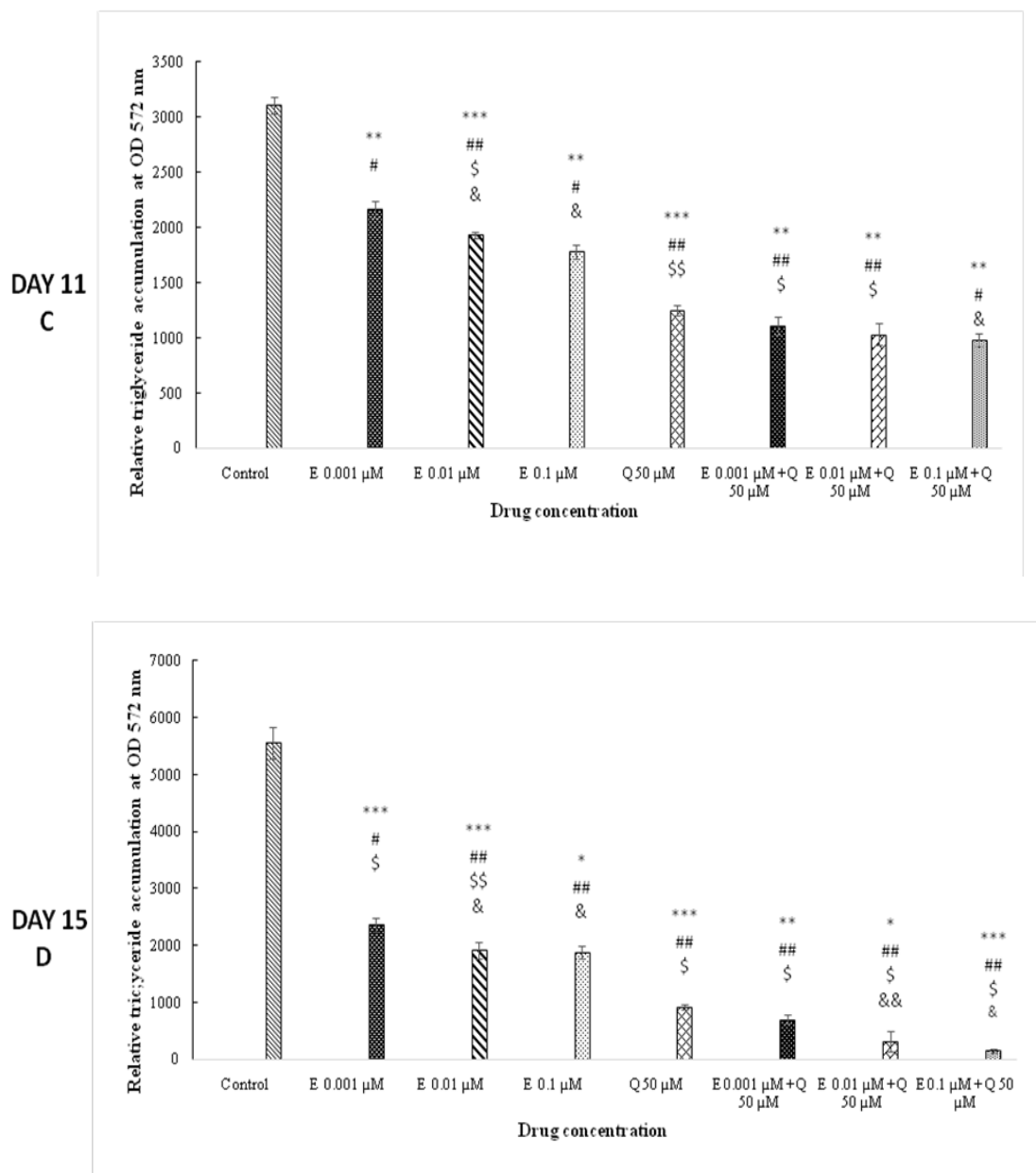


Figure 6.4: (For Images A-D): Bar charts showing the effect of estradiol and quercetin individually and when in combination on the accumulation of triglyceride by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of estradiol and quercetin. Values represent concentration of triglyceride in cell supernatant for the 5th, 8th, 11th and 15th day of culture. Statistical analysis was performed using one-way ANOVA and results are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$, & indicate significance differences within the treatment groups.

The data, presented in figure 6.4 (Taken from Images A-D), show the progression of undifferentiated pre-adipocytes into differentiated mature adipose cells over a period of 15 days either in the presence or absence of indicated concentrations of treatment drugs. Adipose cells that were not treated with any drugs (control) showed a significant progression in the accumulation of triglycerides over 15 days in culture. Image A shows the accumulation of triglycerides on the 5th day of culture. Estradiol at a concentration of 0.001 μM did not exert any change in the lipid content. A small, but significant ($p < 0.05$) decrease was observed at a concentration of 0.01 μM of estradiol. Adipose cells treated with a dosage of 50 μM evoked a significant ($p < 0.01$) reduction in the triglyceride levels. Combination of estradiol and quercetin also decreased the triglyceride concentration in a dose-dependent manner.

The results of triglyceride accumulation were more pronounced on day 8 and day 11 of culture (Image B and C). Estradiol at concentrations of 0.01 μM and 0.1 μM induced a significant ($p < 0.001$ and $p < 0.05$ for day 8 and $p < 0.001$ and $p < 0.01$ for day 11, respectively) decreases in triglyceride levels. Quercetin also inhibited the triglyceride level significantly ($p < 0.001$). When the two drugs were combined together, a significant reduction in triglyceride was measured. Triglyceride levels for day 15 (Image D) showed substantial reductions for adipose cells treated with either estradiol or quercetin alone. This reduction was more pronounced and significant ($p < 0.05$) when the two drugs were combined together.

Overall, the results show that adipose cells treated with either estradiol or quercetin alone and in combination significantly ($p < 0.05$) inhibited lipid formation and attenuated triglyceride accumulation in a dose- and time-dependent manner. This reduction was more prominent when adipose cells were treated with a combination of estradiol and quercetin.

6.3.4 Quantitative measurement of pro-inflammatory and anti-inflammatory adipocytokines

Inflammatory markers of the adipocytokines, including leptin, adiponectin, resistin and interleukin-8, were measured quantitatively using commercially available kits. The data are presented in figures 6.5 -6.8.

6.3.4.1 Quantitative measurement of leptin

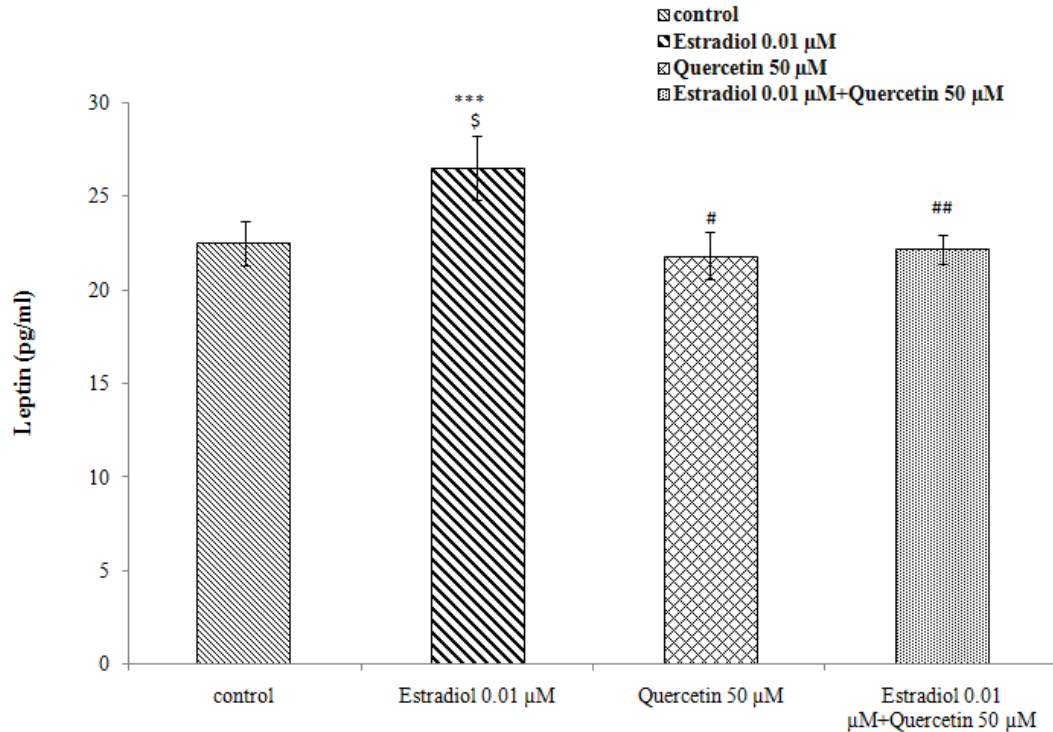


Figure 6.5: Bar charts showing the effect of estradiol and quercetin individually and when in combination on the release of leptin for human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μM estradiol and 50 μM quercetin). Values represent the levels of released leptin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using one-way ANOVA and are presented as mean ± S.D from triplicate set of experiments expressed relative to values from untreated control cells; *p<0.05 **p<0.01 ***p<0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results presented figure 6.5 show a significant elevation in the leptin concentration as compared to control when treated with estradiol alone (p<0.001). However, when the adipocytes were treated with quercetin alone or in combination with estradiol, leptin levels remained largely unaltered.

6.3.4.2 Quantitative measurement of adiponectin

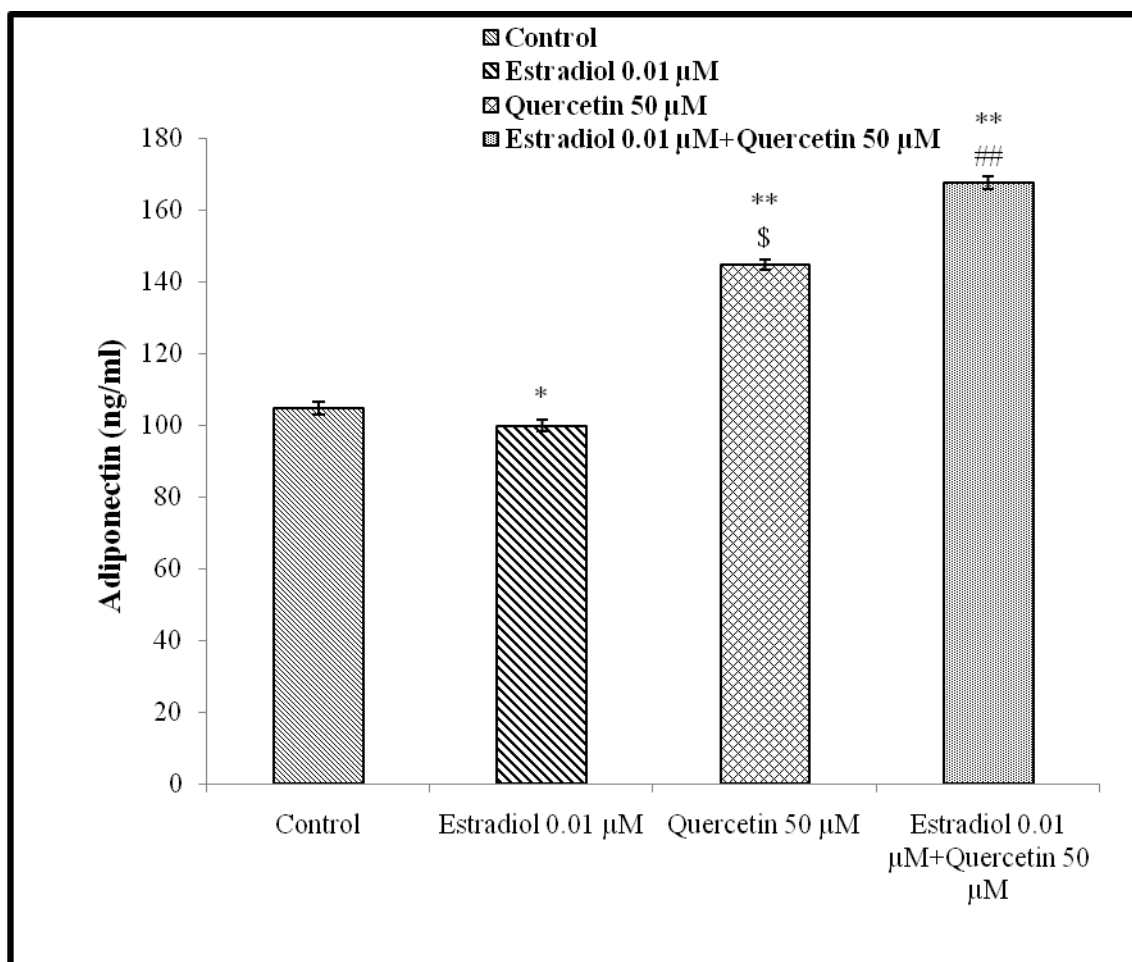


Figure 6.6: Bar charts showing the effect of estradiol and quercetin individually and then in combination on the release of adiponectin by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of released adiponectin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results presented in figure 6.6 show a small but significant reduction in the levels of adiponectin as compared to control when treated with estradiol alone (p <0.05). Significant increases (p <0.01) were observed in the adiponectin concentrations for adipocytes treated with quercetin alone or in combination with estradiol.

6.3.4.3 Quantitative measurement of resistin

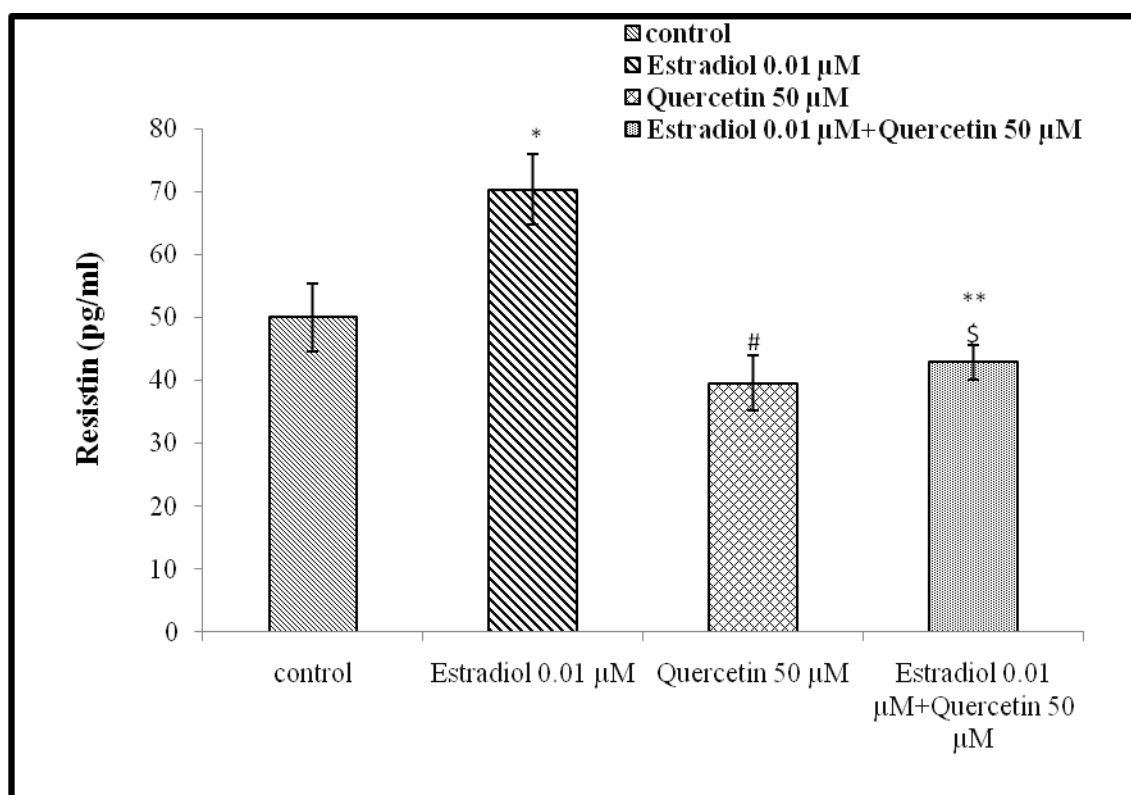


Figure 6.7: Bar graph showing the effect of estradiol and quercetin individually and then in combination on the release of adiponectin by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of released resistin in the cell supernatant for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results presented in figure 6.7 show a significant increase in the resistin concentration as compared to control when treated with estradiol alone (p <0.05). Quercetin alone exerted a substantial reduction in the resistin levels when compared to the untreated or control group. A significant decrease (p <0.01) in resistin level was observed in the adipocytes treated with a combination of estradiol and quercetin when compared to control.

6.3.4.4 Quantitative measurement of Interleukin-8

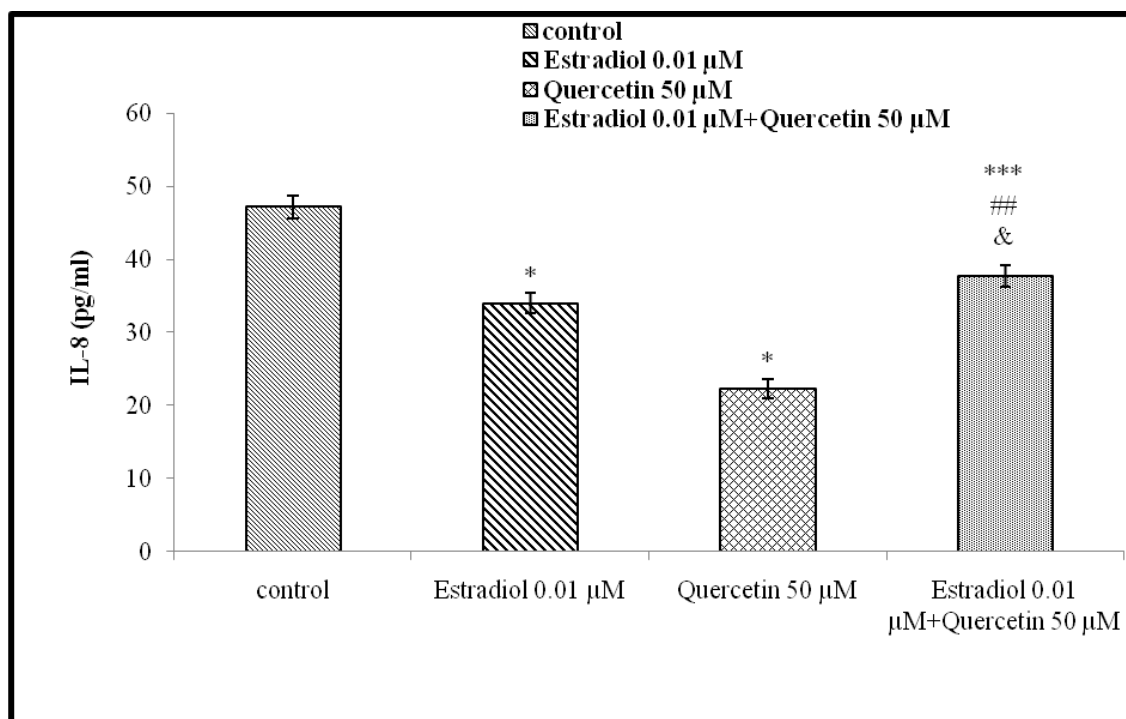


Figure 6.8: Bar charts showing the effect of estradiol and quercetin individually and then in combination on the release of interleukin-8 by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of released interleukin-8 in the cell supernatant for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ indicate significant differences within the treatment groups.

The results presented in figure 6.8 show a significant (p <0.05) reduction in the levels of interleukin-8 as compared to control when treated with either estradiol or quercetin alone. Similarly, there was a significant (p <0.001) decrease in IL-8 level when estradiol was combined with quercetin.

6.3.5 Quantitative measurement of markers of oxidative stress

Markers of oxidative stress including nitric oxide, superoxide dismutase, glutathione and catalase were measured quantitatively using commercially available kits. The data are presented in figures 6.9 -6.12.

6.3.5.1 Quantitative measurement of Nitric oxide

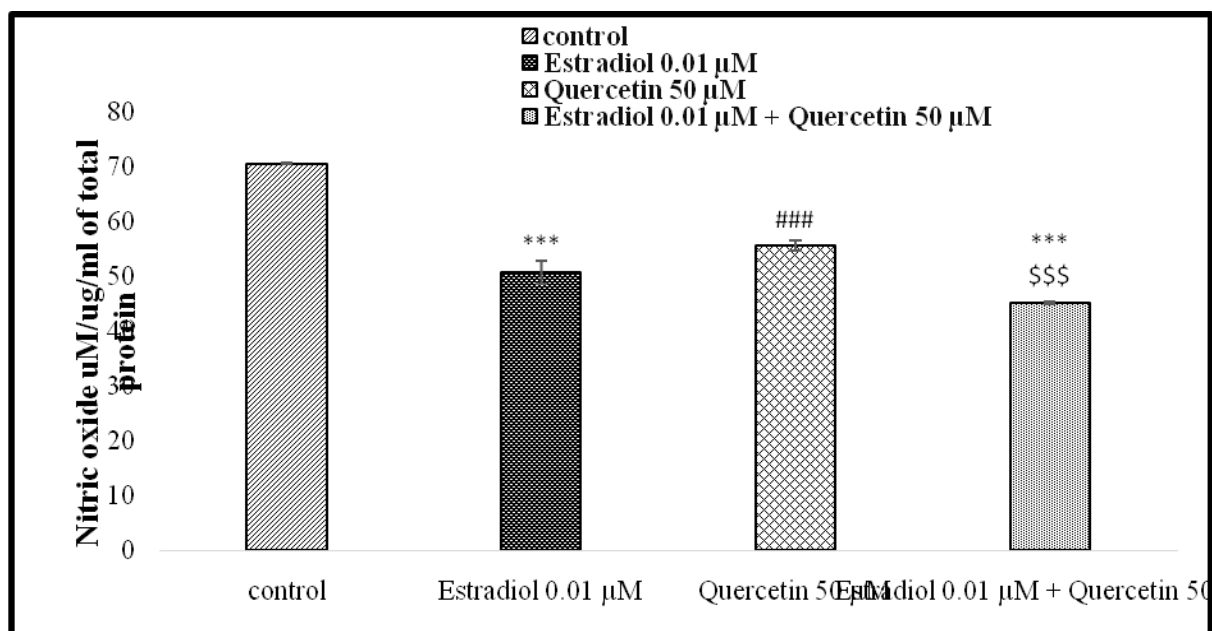


Figure 6.9: Bar charts showing the effect of estradiol and quercetin individually and then in combination on the release of nitric oxide by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of nitric oxide in the cell lysates for the 8th day of culture. Statistical analysis was performed using one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ represents significance difference within the treatment groups.

The data from figure 6.9 show significant reduction in the nitric oxide concentration for adipose cells treated with estradiol or quercetin alone (p <0.05 and p <0.01 respectively) as compared to control. When the two drugs were combined, further significant decrease (p <0.01) in the nitric oxide levels were observed.

6.3.5.2 Quantitative measurement of catalase

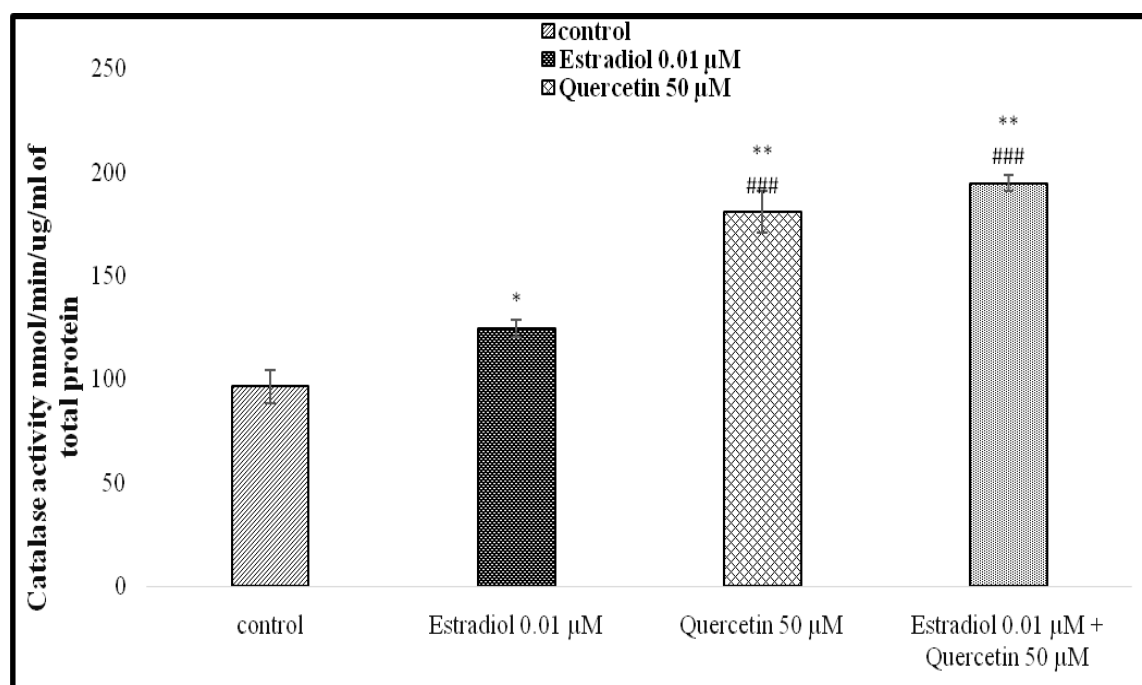


Figure 6.10: Bar graphs showing the effect of estradiol and quercetin individually and then in combination on the catalase activity by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of catalase enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ represent significance difference within the treatment groups.

The data from figure 6.10 represent significant increase in the catalase activity for adipose cells treated with either estradiol or quercetin alone (p <0.05 and p <0.01 respectively) as compared to control. This increase is further enhanced significantly (p <0.01) when the two drugs are combined together.

6.3.5.3 Quantitative measurement of Superoxide dismutase

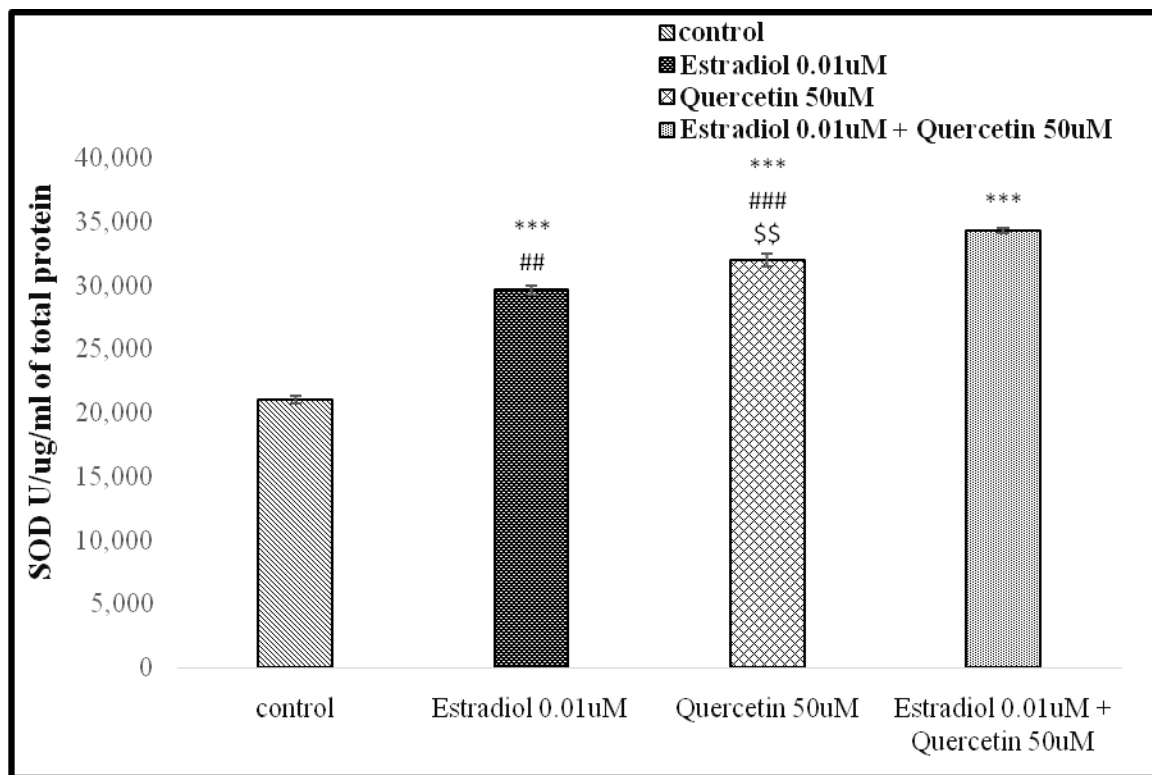


Figure 6.11: Bar graphs showing the effect of estradiol and quercetin individually and then in combination on the superoxide dismutase activity by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin). Values represent concentrations of superoxide dismutase enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using one-way ANOVA for equality of variances and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ represents significance difference within the treatment groups.

The data from figure 6.11 show significant increase (p <0.05) in SOD levels for adipose cells treated with either estradiol or quercetin alone or in combination as compared to control.

6.3.5.4 Quantitative measurement of glutathione

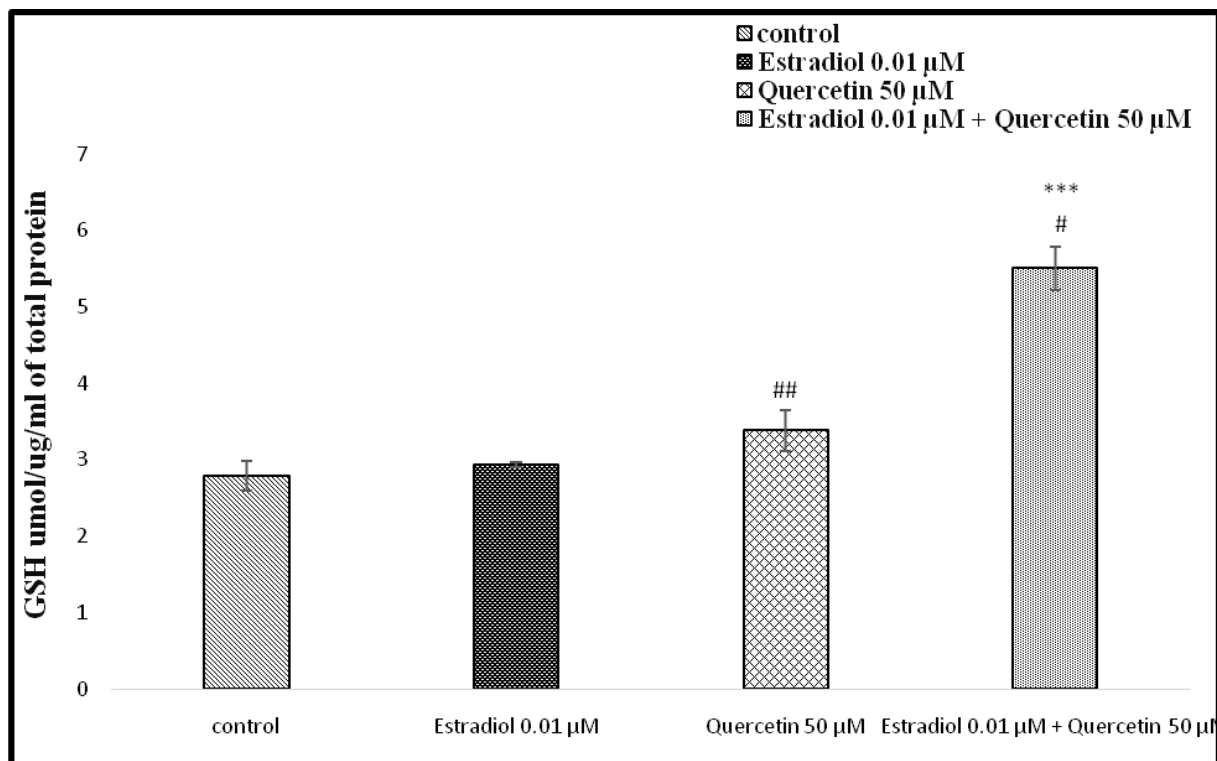


Figure 6.12: Bar graph showing the effect of estradiol and quercetin individually and then in combination on the concentration of glutathione by human adipocytes in culture. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μM estradiol and 50 μM quercetin). Values represent concentrations of glutathione enzyme in the cell lysates for the 8th day of culture. Statistical analysis was performed using one-way ANOVA for equality of variances and are presented as means ± S.D from triplicate set of experiments expressed relative to values from untreated control cells; *p<0.05 **p<0.01 ***p<0.001 compared to the control. #, \$ represents significance difference within the treatment groups.

The results from figure 6.12 show significant increase (p<0.01) in glutathione activity for adipose cells treated with a combination of estradiol and quercetin. Only a slight increase (not significant) was observed in the glutathione levels of adipose cells treated individually with either estradiol or quercetin.

6.3.6 Immuno-fluorescence staining using adipogenic marker antibodies

This series of experiment was designed to demonstrate both the presence and the subcellular localization of crucial protein markers involved in the process o adipogenesis. Briefly, adipose cells were grown and then treated with either estradiol or quercetin alone and in combination, fixed and stained directly in 96 well black-coloured plates using primary antibodies for apecific adipogenic markers. Goat anti-rabbit fluorochrome-conjugated secondary antibody, Alexa Fluor was used to stain sub-cellular proteins while DAPI was used to stain the nuclei.

6.3.6.1 Morphological and quantitative measurement of Perilipin

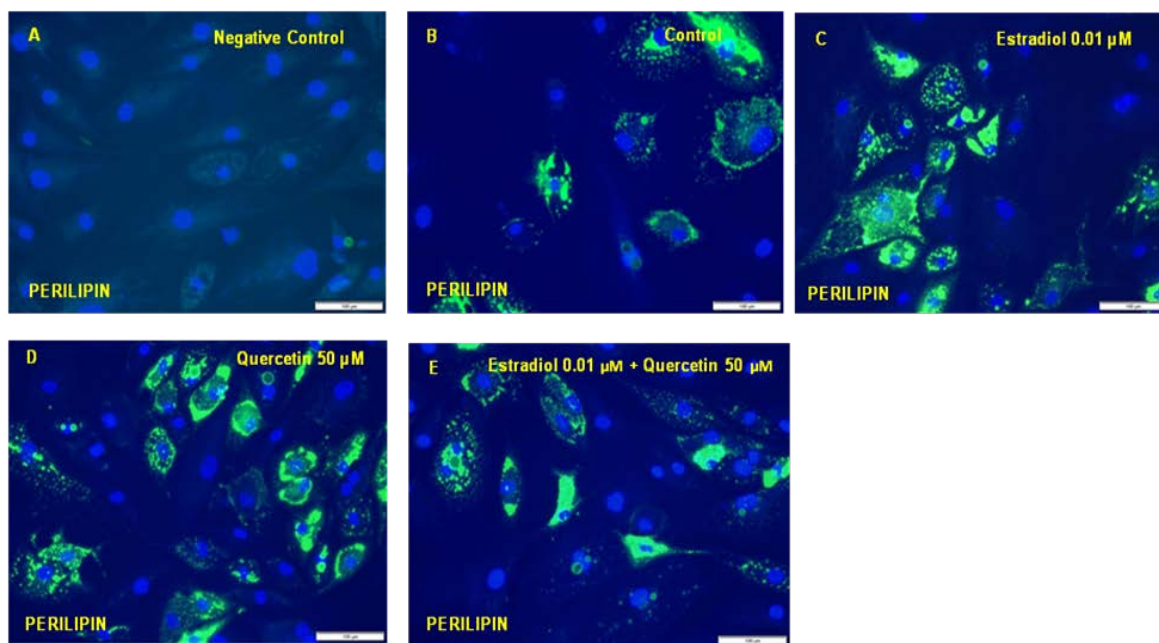


Figure 6.13: Images A-E showing expression of perilipin adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Perilipin (green) was detected using perilipin primary antibody diluted at 1:200 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M. Images are typical of triplicate series of experiments.

A). Negative control without the primary antibody.

B). Differentiated adipose cells without any drug treatment (Control).

C). Differentiated adipose cells treated with 0.01 μM estradiol.

D). Differentiated adipose cells treated with 50 μM quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μM estradiol and 50 μM quercetin. These images are typical of 3 different experiments.

Morphological results obtained from figure 6.13 (Images A-E) show increased expression of perilipin in adipose cells treated with 0.01 μM estradiol as compared to control. Quercetin also induced increased levels of perilipin which subsequently decreased with the combination of the two drugs together.

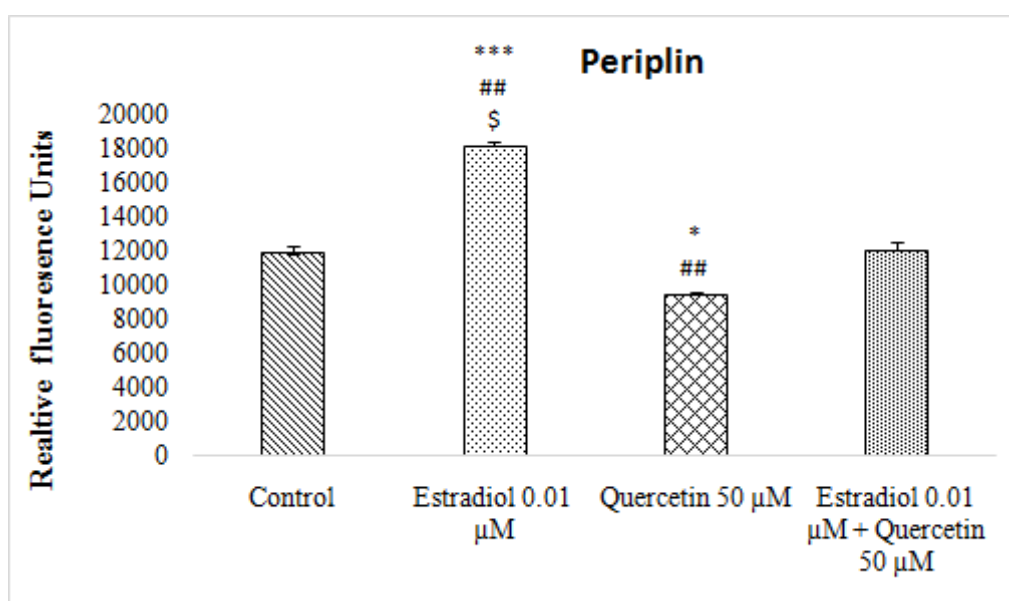


Figure 6.14: Bar graphs showing the quantitative measurement of perilipin in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μM estradiol and 50 μM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. #, \$ represents significance difference within the treatment groups.

The results from figure 6.14 show significant ($p<0.001$) increase in the level of perilipin for adipose cells treated with estradiol as compared to control. Treatment with quercetin alone or in combination with estradiol did not show much increase in the perilipin levels and was almost comparable to control.

6.3.6.2 Morphological and quantitative measurement of Fatty acid synthase

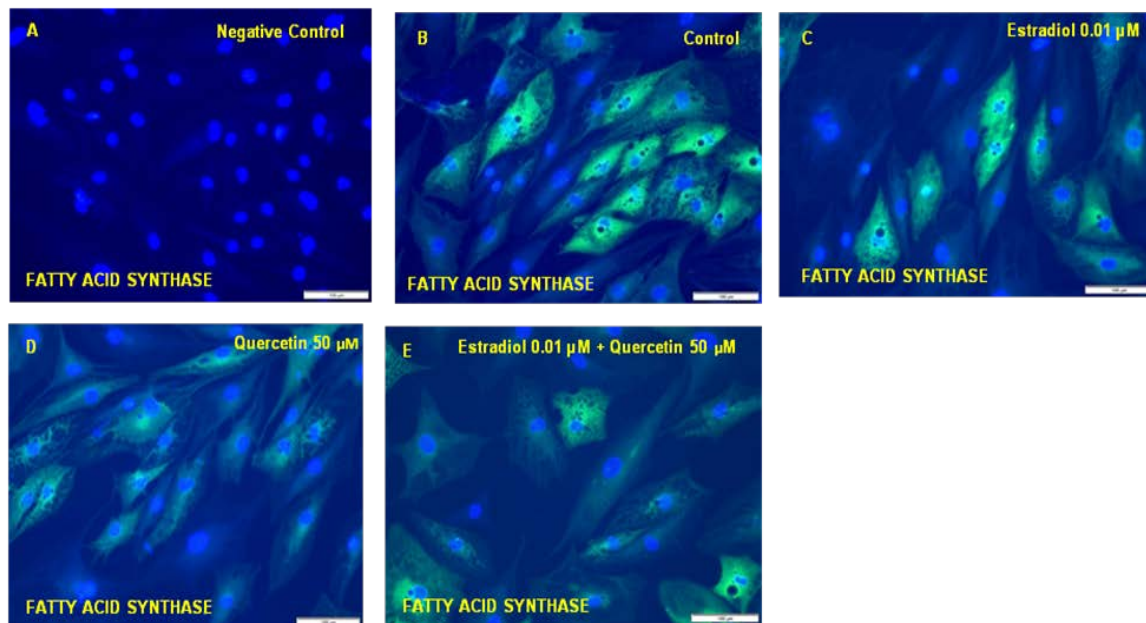


Figure 6.15: Images A-E showing expression of fatty acid synthase adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Fatty acid synthase (green) was detected using fatty acid synthase primary antibody diluted at 1:50 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μM . Images are typical of triplicate series of experiments.

- A). Negative control without the primary antibody.
- B). Differentiated adipose cells without any drug treatment (Control).
- C). Differentiated adipose cells treated with 0.01 μM estradiol.
- D). Differentiated adipose cells treated with 50 μM quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μM estradiol and 50 μM quercetin. These images are typical of 3 different experiments.

The results from figure 6.15 show a significant ($p < 0.05$) decrease in the expression of fatty acid synthase marker for adipose cells treated with either estradiol or quercetin alone as compared to control. This decrease was more pronounced ($p < 0.05$) when adipose cells were treated with a combination of these two drugs.

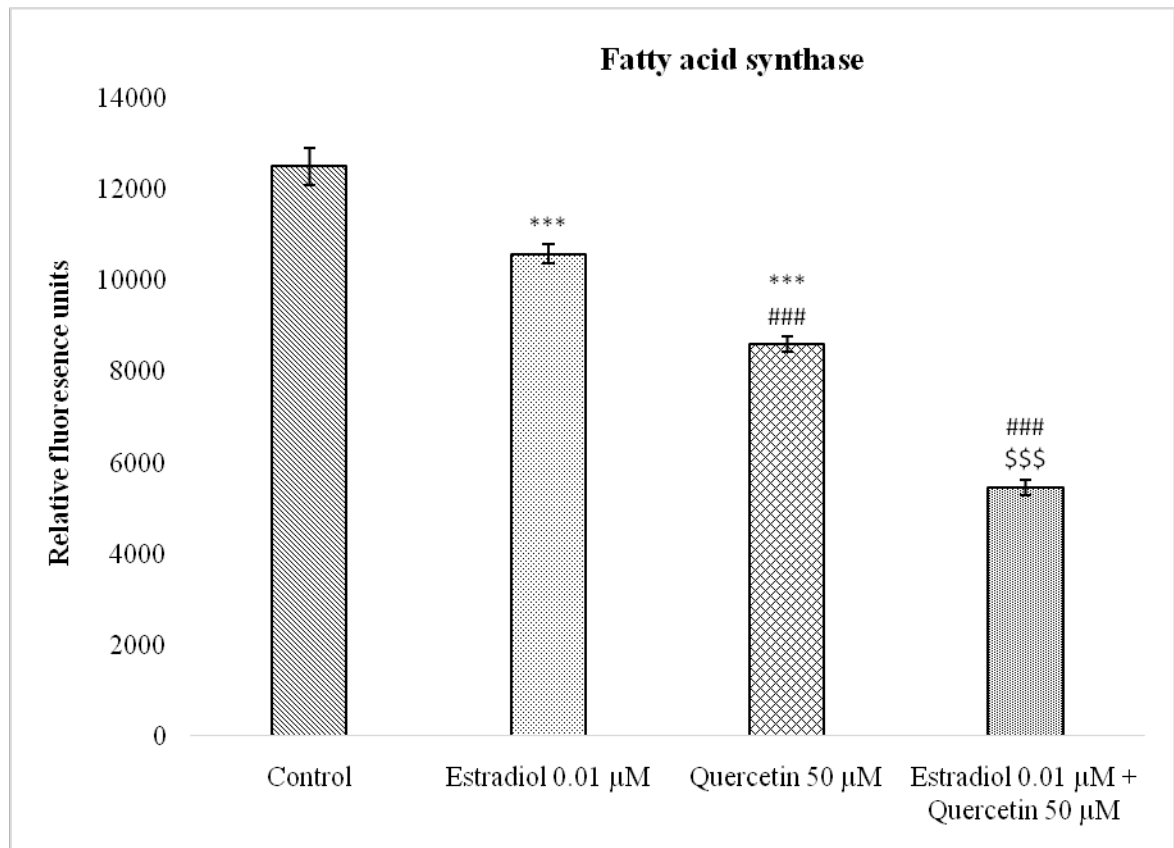


Figure 6.16: Bar graph showing the quantitative measurement of periplin in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μM estradiol and 50 μM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$

*** $p < 0.001$ compared to the control. #, \$ represents significance difference within the treatment groups.

The data from figure 6.16 show significant ($p < 0.001$) decrease in the level of fatty acid synthase for adipose cells treated with either estradiol or quercetin as compared to control. This reduction in fatty acid synthase level was more significantly reduced ($p < 0.001$) when adipose cells were treated with a combination of the two drugs together.

6.3.6.3 Morphological and quantitative measurement of FABP4/aP2

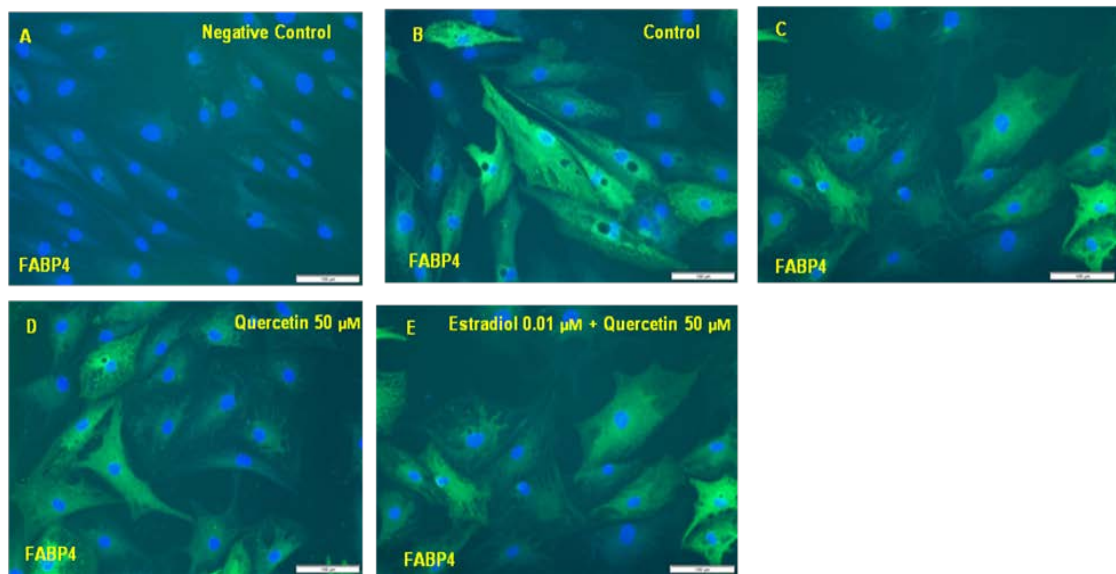


Figure 6.17: Images A-E showing expression of FABP4/aP2 adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. FABP4/aP2 (green) was detected using FABP4/aP2 primary antibody diluted at 1:200 concentrations. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μM . The images are typical of triplicate set of experiments.

- A). Negative control without the primary antibody.
- B). Differentiated adipose cells without any drug treatment (Control).
- C). Differentiated adipose cells treated with 0.01 μM estradiol.
- D). Differentiated adipose cells treated with 50 μM quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μM estradiol and 50 μM quercetin. These images are typical of 3 different experiments.

The morphological observations obtained from figure 6.17 show a decrease in the expression of FABP4/aP2 marker when the adipose cells were treated with either estradiol or quercetin alone or in combination.

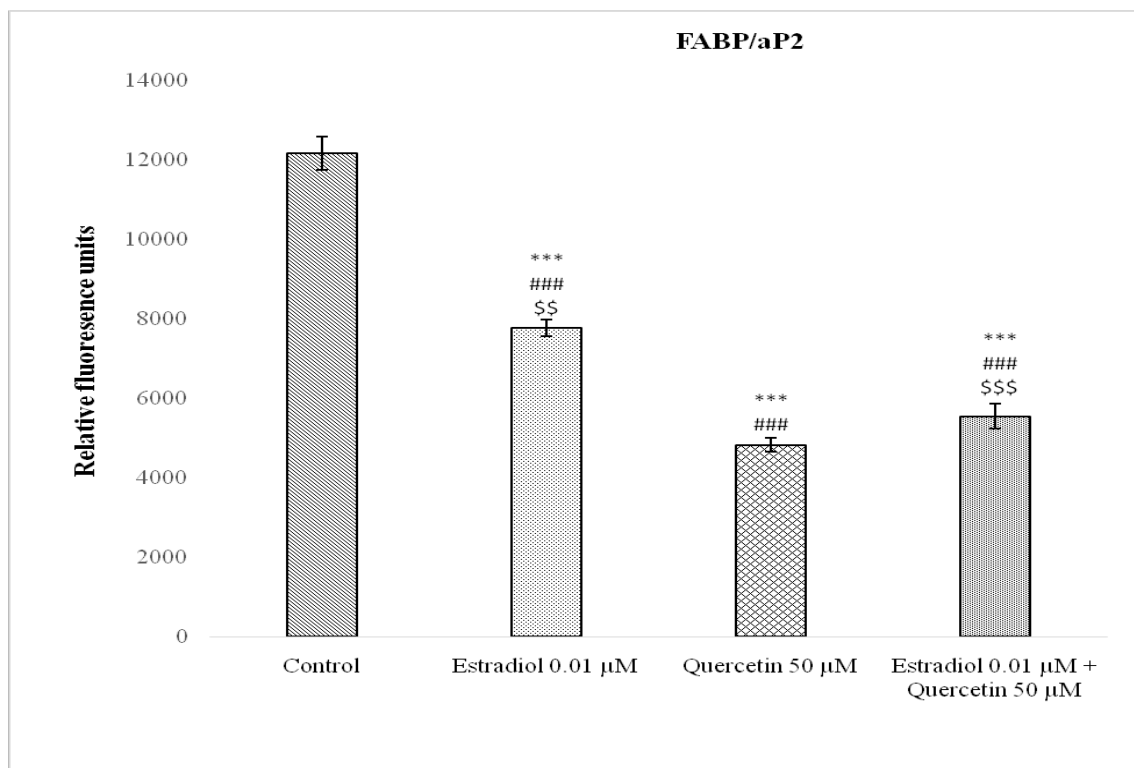


Figure 6.18: Bar graphs showing the quantitative measurement of FABP4/aP2 in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μM estradiol and 50 μM quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. #, \$ represents significance difference within the treatment groups.

The results from figure 6.18 show a significant reduction ($p < 0.001$) in the level of FABP4/aP2 when adipose cells were treated with either estradiol or quercetin alone. Also, a significant reduction ($p < 0.001$) was measured in the FABP4/aP2 level when the adipose cells were treated with a combination of both estradiol and quercetin together.

7.3.6.4 Morphological and quantitative measurement of acetyl coenzyme-A carboxylase

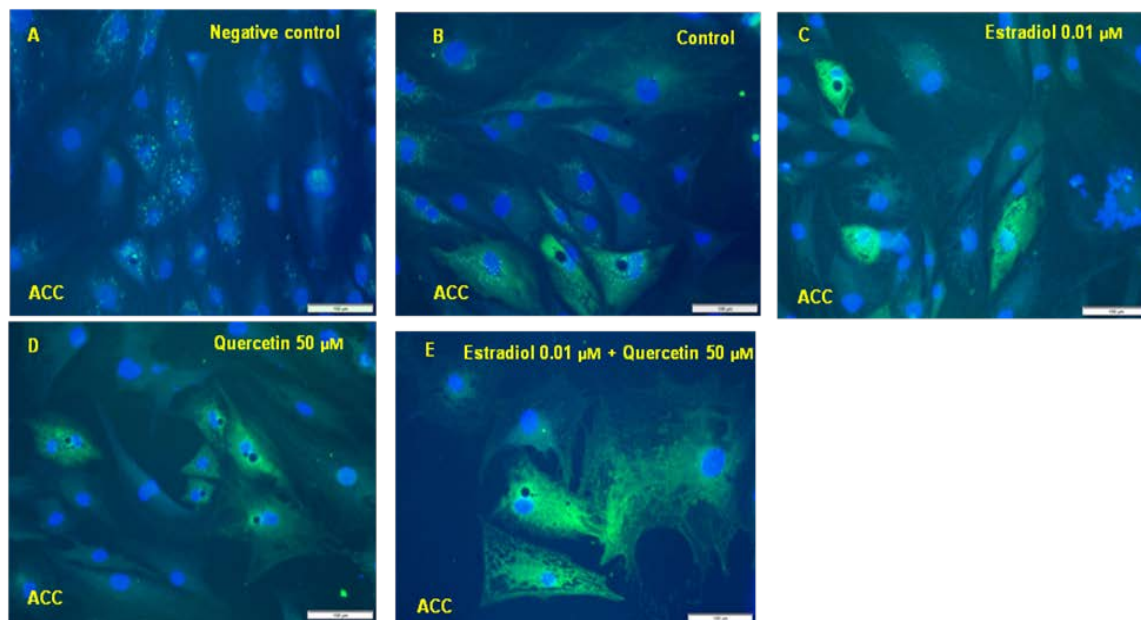


Figure 6.19: Images A-E showing expression of acetyl coenzyme-A carboxylase adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. Acetyl coenzyme-A carboxylase (green) was detected using acetyl coenzyme-A carboxylase primary antibody diluted at 1:200 concentrations. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μM . These images are typical of triplicate set of experiments.

A). Negative control without the primary antibody.

B). Differentiated adipose cells without any drug treatment (Control).

C). Differentiated adipose cells treated with 0.01 μM estradiol.

D). Differentiated adipose cells treated with 50 μM quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μM estradiol and 50 μM quercetin. These images are typical of 3 different experiments.

Morphological data from figure 6.19 shows that both estradiol and quercetin evoke a reduction in the acetyl coenzyme-A carboxylase expression when adipose cells were treated with estradiol or quercetin alone or in combination.

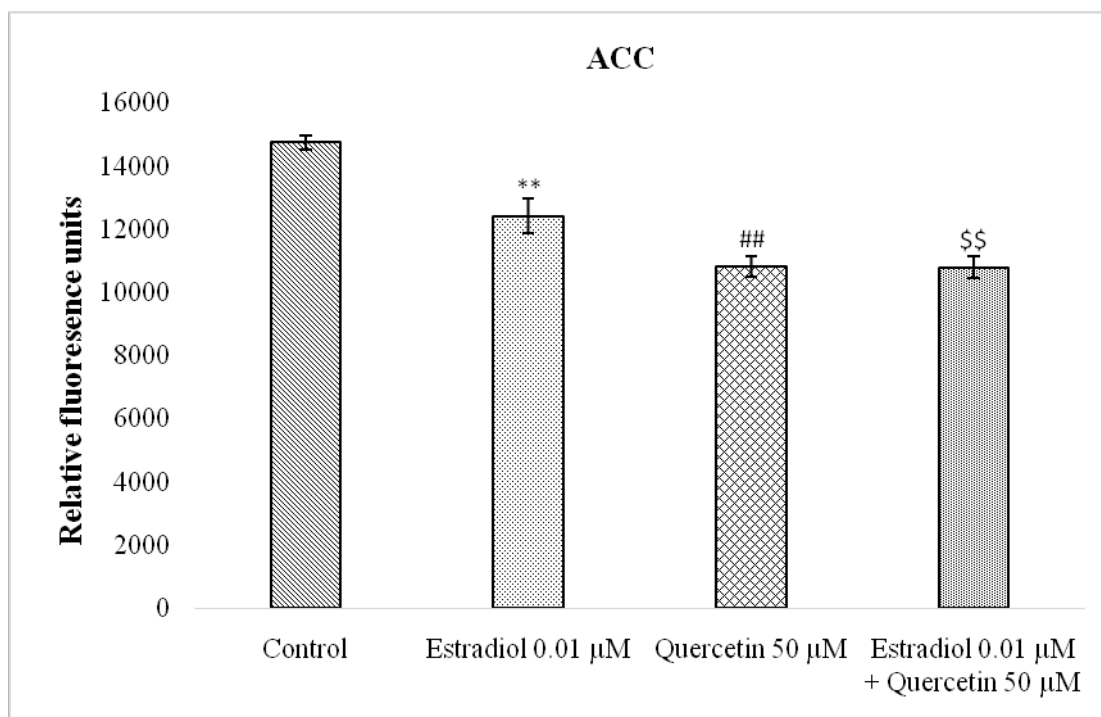


Figure 6.20: Bar graph showing the quantitative measurement of acetyl coenzyme-A carboxylase in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ represents significance difference within the treatment groups.

The results from figure 6.20 show a significant decrease (p <0.01) in the levels of acetyl coenzyme-A carboxylase when adipose cells were treated with either estradiol or quercetin alone. However, when the two drugs were combined together, a non-significant reduction was observed in comparison to the control.

6.3.6.5 Morphological and quantitative measurement of PPAR γ

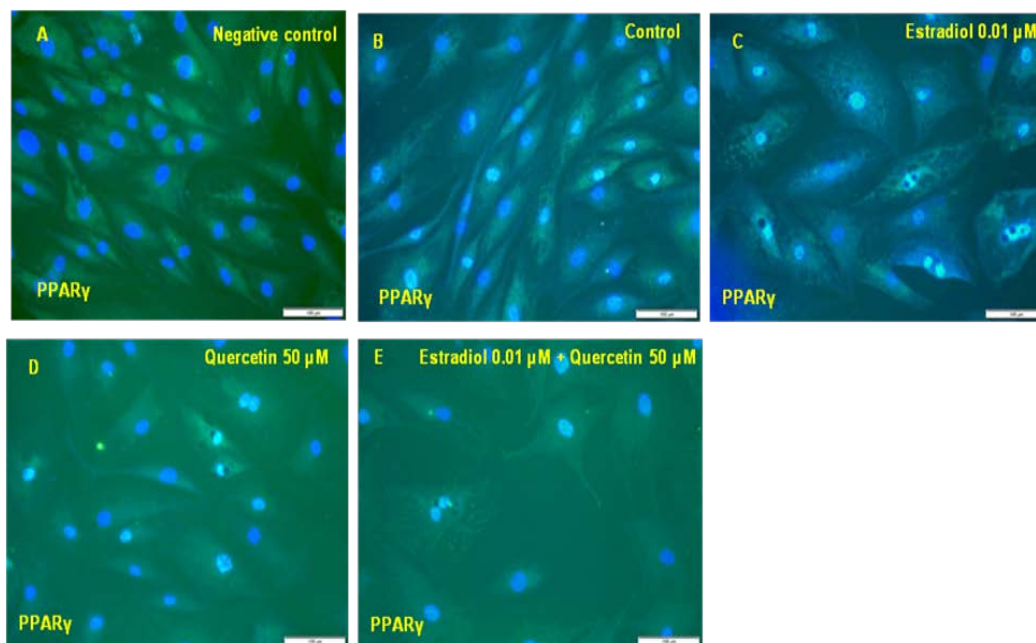


Figure 6.21: Images A-E showing expression of PPAR γ adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. PPAR γ (green) was detected using PPAR γ primary antibody diluted at 1:200 concentrations. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M. Images are typical of 3 different experiments.

A). Negative control without the primary antibody.

B). Differentiated adipose cells without any drug treatment (Control).

C). Differentiated adipose cells treated with 0.01 μ M estradiol.

D). Differentiated adipose cells treated with 50 μ M quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μ M estradiol and 50 μ M quercetin. These images are typical of 3 different experiments.

The results from figure 7.21 show an attenuated expression of the PPAR γ marker for adipose cells treated with either estradiol or quercetin alone or in combination.

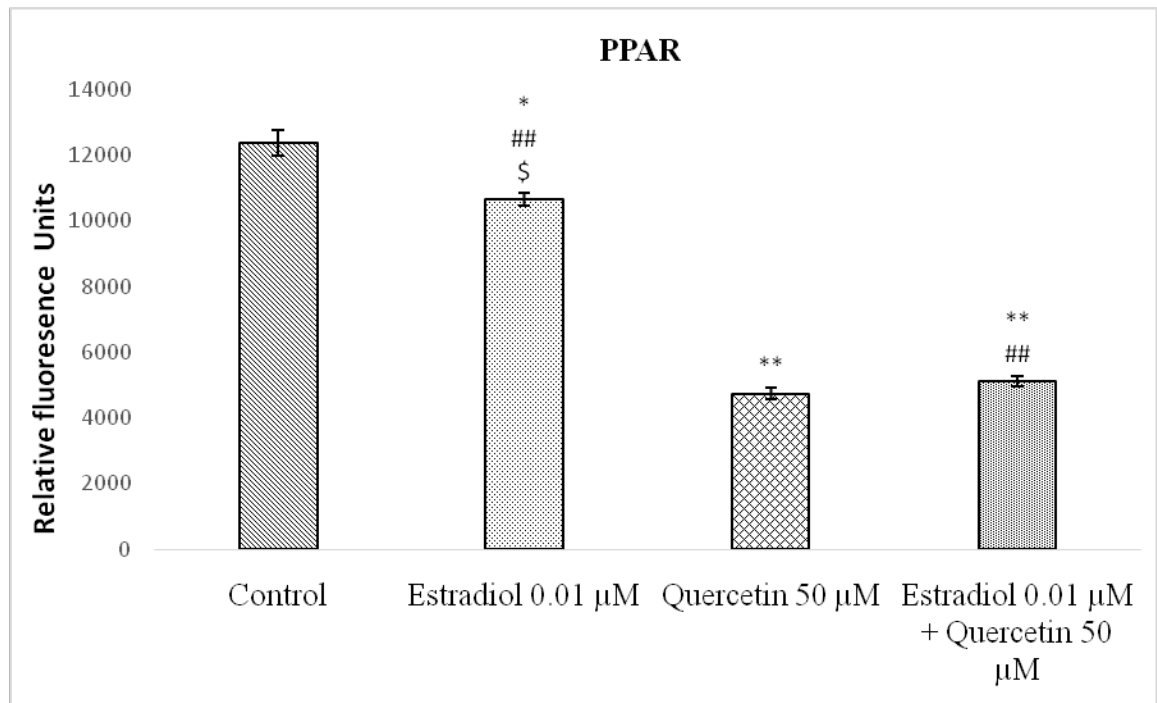


Figure 6.22: Bar graph showing the quantitative measurement of PPAR γ in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as means of \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the control. #, \$ represent significant difference within the treatment groups.

The data from figure 6.22 show significant ($p < 0.05$, $p < 0.01$) attenuation in the PPAR γ expression for adipose cells treated with either estradiol or quercetin respectively. A significant decrease ($p < 0.01$) in PPAR γ was measured for adipose cells treated with a combination of estradiol and quercetin in comparison to control.

6.3.6.6 Morphological and quantitative measurement of CCAAT/enhancer-binding protein (CEBP)

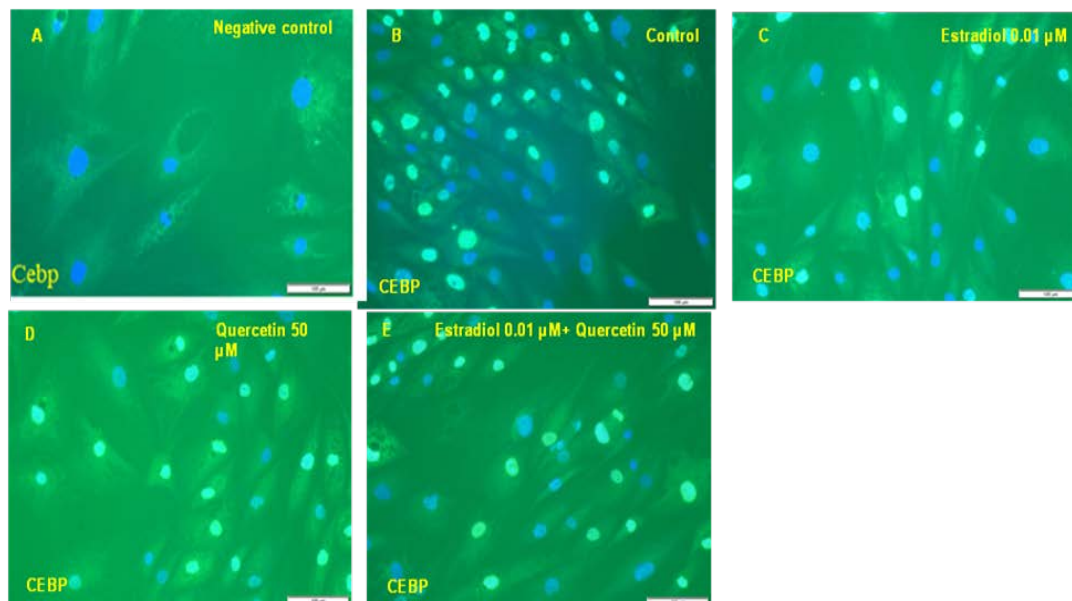


Figure 6.23: Images A-E showing expression of CCAAT/enhancer-binding protein adipogenic marker in human subcutaneous adipose cells differentiated for a period of 8 days in the presence of treatment drugs using immuno-fluorescence dual staining. CCAAT/enhancer-binding protein (green) was detected using CCAAT/enhancer-binding protein primary antibody diluted at 1:200 concentration. Nuclei (blue) were stained with DAPI. Cells were imaged at 20X magnification with a scale bar of 100 μ M. The images are typical of 3 different experiments.

A). Negative control without the primary antibody.

B). Differentiated adipose cells without any drug treatment (Control).

C). Differentiated adipose cells treated with 0.01 μ M estradiol.

D). Differentiated adipose cells treated with 50 μ M quercetin.

E). Differentiated adipose cells treated with a combination of 0.01 μ M estradiol and 50 μ M quercetin. These images are typical of 3 different experiments.

Morphological data presented in figure 6.23 show a repression in the CCAAT/enhancer-binding protein marker when adipose cells were treated with either estradiol or quercetin alone or in combination.

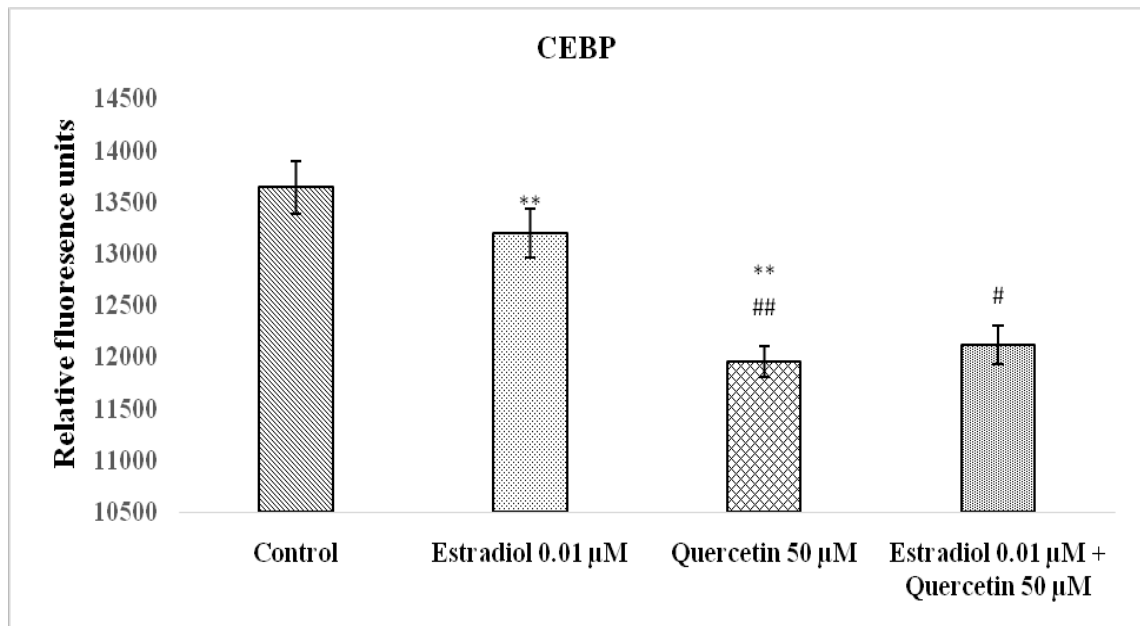


Figure 6.24: Bar graphs showing the quantitative measurement of CCAAT/enhancer-binding protein in human adipose cells treated with estradiol and quercetin. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol and 50 μ M quercetin) individually and then in combination. Fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm for adipose cells on day 8 in culture. Statistical analysis was performed using a one-way ANOVA and are presented as mean \pm S.D from triplicate set of experiments expressed relative to values from untreated control cells; * p <0.05 ** p <0.01 *** p <0.001 compared to the control. #, \$ represents significance difference within the treatment groups.

The results presented in figure 6.24 show a significant (p < 0.01) decrease in CCAAT/enhancer-binding protein when adipose cells were treated with either estradiol or quercetin alone in comparison to the control. However, when adipose cells were treated with a combination of estradiol and quercetin, the results show a non-significant reduction (p >0.05) as compared to control.

6.3.7 Gene expression analysis using RT-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to determine the level of mRNA expression for adipocyte-specific genes and major adipogenic transcriptional factors (PPAR- γ , SREBP-1, C/EBP, LPL, aP2 and FAS) using specific primers and commercially available kits. Briefly, on day 8 after differentiation, total RNA was extracted from cultured cells and reverse transcribed. PCR analyses were then performed on cDNA preparation aliquots to detect mRNA expression. The amount of cDNA templates used for PCR was determined within the linear range. A 10 μ L aliquot from each PCR reaction was electrophoresed in a 1.5% agarose gel containing 0.2 μ g/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. The levels of adipogenic marker genes were expressed after normalization with the β -actin signal from the same sample.

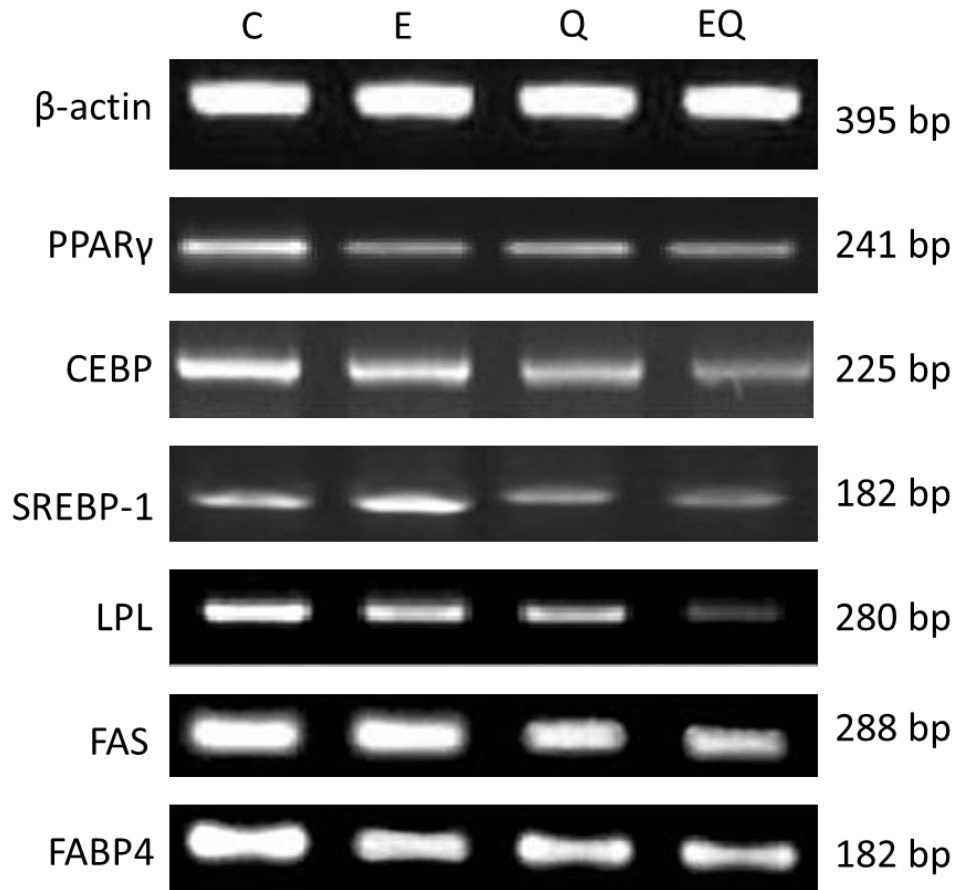


Figure 6.25: RT-PCR analysis of the mRNA expression of adipogenic markers. Human pre-adipocytes were differentiated in the presence of indicated concentrations of drugs (0.01 μ M estradiol, 50 μ M quercetin) individually and then in combination. After 8 days, the mRNA

expression levels of PPAR γ , C/EBP α , SREBP-1, LPL, FABP4 and FAS were measured using RT-PCR. The PCR gel are typical of 3 different experiments.

The data from figure 6.25 show that the expression of adipogenic markers is significantly ($p < 0.05$) reduced for adipose cells treated with either estradiol or quercetin when compared to control. This reduction is more pronounced when both the drugs are combined.

6.4 Discussion

It is now well acknowledged that adipose tissue is a true endocrine-secreting organ, releasing such hormones as cytokines, chemokines, growth factors and others which in turn can influence metabolism. It is also recognized as a target for sex steroids since sex steroid receptors are expressed both in rat and human adipose tissues. In particular, 17 β -estradiol (E2) has been recognized as a major factor in regulating adipose tissue metabolism in females. It has been reported that ovary-ectomy in rodents leads to weight gain, primarily in the form of adipose tissue, which is reversed by physiologic estrogen replacement (Jeong et al., 2004; Jeong et al., 2012). Loss of circulating estrogen has been shown to be associated with an increase in adiposity during menopause, whereas postmenopausal women who receive estrogen replacement therapy do not display the characteristic abdominal weight gain pattern usually associated with menopause (Carani et al., 1997). Estradiol also plays an important role in regulating adipocyte differentiation and development. It has been reported to repress adipogenic differentiation and maturation via an estrogen receptor (ER)-dependent mechanism in human and mouse bone marrow stromal cells (Okazaki et al., 2002). In parallel, several plant compounds have been reported to mimic estrogenic properties. The phytoestrogen genistein, which has high affinity for estrogen receptors has been demonstrated to inhibit adipocyte differentiation, lipid accumulation, and the expression of adipocyte-specific genes in primary human adipocytes (Heim et al., 2004; Park et al., 2009). Estradiol was also reported to stimulate the proliferation of human pre-adipocytes, which can remain undifferentiated cells, into adipocytes (Roncari et al., 1978).

In order to understand the effect of 17 β -estradiol (E2) on the differentiation of human subcutaneous adipose cells and determine the optimal dose concentration, pre-adipocytes were treated with pharmacological concentrations of 17 β -estradiol and the viability of the cells was determined by LDH assay. Initial results show that 17 β -estradiol treatment up to a dosage

concentration of 0.1 μM had no significant effect on cell viability on day 3 of culture. However, on day 15, the results show that either estradiol at lower concentrations (0.001 μM and 0.01 μM) or quercetin at 50 μM did not affect the viability rate of the cells. However, at a concentration of 0.1 μM of estradiol either alone or in combination with 50 μM of quercetin, significant decreases in cell viability ($p < 0.05$ and $p < 0.01$) respectively, were observed.

These doses were also tested to assess their effects on triglyceride accumulation. The triglyceride content of intracellular fat droplets, as evidenced by adipo-red staining and quantitative measurement using fluorimeter, was attenuated by 17 β -estradiol in both a dose-dependent and time dependent manner suggesting that 17 β -estradiol treatment reduces triglyceride accumulation in human subcutaneous pre-adipocyte cells undergoing differentiation. The present literature indicates that estradiol (E2) can have an effect on adipocyte differentiation, although the reports in this area are not entirely consistent. Studies with rat pre-adipocytes indicate that E2 stimulates differentiation of these cells into adipocytes (Dieudonne et al., 2000). In contrast to these findings, other results have indicated that E2 can inhibit differentiation of adipocytes in the 3T3-L1 cell line (Lea-Currie et al., 1999). The inhibitory effects of estrogen on adipocyte differentiation reported in this study are consistent with numerous other reports (Okazaki et al., 2002; Heim et al., 2004; Dang et al., 2009) showing that estrogen inhibits adipogenesis in primary bone marrow stromal cell cultures or bone marrow stroma cell lines.

Based on these preliminary studies, optimal concentrations of 0.01 μM of estradiol and 50 μM of quercetin were chosen for subsequent experiments. The finding that obesity is associated with a systemic low-grade inflammatory state and that inflammation mediates many of the pathological consequences of obesity has opened up new avenues to the study of the interactions between metabolic and immunological processes (Mathis et al., 2011). Besides its role as a fat storing organ, adipose tissue is well recognized as an endocrine organ secreting a number of adipocytokines such as adiponectin, leptin, resistin and IL-8 (Ordovas et al., 2008). Some of these factors have been reported to be the culprit of low-grade inflammation in obesity (Kershaw et al., 2004; Lafontan, 2004). Since estrogens have a role in adipocyte metabolism, as well as fat distribution, studying the modulation of adipokine production and its release may be of patho-physiological importance.

The next series of experiments was thus conducted to study the role of either estradiol or quercetin alone or in combination on adipose cells in vitro on some of these inflammatory

markers. Several previously conducted studies have shown that estrogen may contribute to the prevention and/or improvement of obesity-related metabolic syndromes by altering the production of adipocytokines in rodents, monkeys and humans (Hong et al., 2007; Kafkas et al., 2012; Shaw et al., 2013). One of the adipo-cytokines that shows a strong correlation with estrogen is leptin. It is a key metabolic hormone involved in the central regulation of metabolism and transfers a catabolic signal to the brain to inhibit food intake and increase energy expenditure (Ahima et al., 1999; Elmquist et al., 1999). Data from the present study show increased leptin levels when adipose cells were treated with estradiol in comparison to non-treated cells. These results are in parallel with other studies showing increased levels of leptin in adipose tissue when treated with estrogen (Shimizu et al., 1997; Fungfuang et al., 2013). Leptin levels remained unaltered when adipose cells were treated with either quercetin alone or in combination with estradiol. Adiponectin is another insulin-sensitive hormone and plays a central role in lipid and glucose metabolism. However, unlike other adipokines, the plasma level of adiponectin is inversely correlated with body mass index. In the present study, 17 β -estradiol dramatically suppressed the level of adiponectin. This decrease in adiponectin concentration seems to be associated with insulin resistance and other related disorders (Kowalska et al., 2008). In another study on adipocytes, estrogen treatment suppressed adiponectin mRNA and protein expression, and this effect was blocked by treatment with an estrogen antagonist (Combs et al., 2003). It may therefore be indicated that estradiol may exert protective effects against insulin resistance and obesity through the regulation of adiponectin production. Treatment of adipose cells with quercetin alone or in combination with estradiol significantly increased adiponectin levels demonstrating the anti-inflammatory properties of quercetin.

Resistin is a cysteine-rich hormone that was first isolated from adipose tissues and found to link obesity to T2DM in rodents (Steppan et al., 2001). The involvement of resistin in obesity and insulin resistance in humans is still controversial. Several studies have shown no relationship of resistin gene expression with body weight or insulin resistance (Koerner et al., 2005), while others found that resistin mRNA expression in adipose tissues of obese humans is higher than that in normal subjects (McTernan et al., 2002). A single-nucleotide polymorphism in the resistin gene promoter was reported to be associated with obesity and diabetes (Engert et al., 2002; Pizzuti et al., 2002) and that the plasma resistin levels were elevated in patients with obesity (Degawa-Yamauchi et al., 2003) and T2DM (Youn et al., 2004). The present study showed copiously elevated resistin concentration levels when adipose cells were treated with estradiol.

This increase in resistin concentration may be attributed to the expression of CEBP α which acts as a transcriptional activator of resistin. This implication is also indirectly supported by the findings that a functional C/EBP-binding site was found in the proximal resistin promoter of both murine and human adipocytes to be necessary for the stimulation of transcription from resistin promoter (Hartman et al., 2002; Song et al., 2002; Seo et al., 2003) and that endogenous C/EBP was bound to the resistin promoter in adipocytes in association with p300 and CREB-binding protein, which are viewed as co-activators involved in ER activation (Nilsson et al., 2001). Adipose cells treated with quercetin alone or in combination with estradiol showed decreased resistin concentration in adipose cells.

Although not much studied, IL-8 has been reported to be produced and released from human adipose tissue in regulated manner (Bruun et al., 2001; Gerhardt et al., 2001). Besides the participation in inflammatory processes, its involvement has been suggested in the development of obesity-associated insulin resistance (Kern et al., 2001), atherosclerosis and cardiovascular disease (Boisvert et al., 2000; Schieffer et al., 2000). The present study showed significant reduction in IL-8 levels when adipose cells were treated with either estradiol or quercetin alone or in combination, suggesting the protective effects of these two drugs on the development of metabolic syndrome.

Oxidative stress reflects an imbalance between systemic production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (Lu et al., 2009). Furthermore, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in the normal mechanisms of cellular signaling. In humans, oxidative stress is thought to be involved in the pathogenesis and development of metabolic syndrome. The present study, on human adipocytes in vitro, shows that both estradiol and quercetin can decrease the nitric oxide levels and increase the anti-oxidant enzyme activities suggesting a protective role of these drugs in adipose tissue.

The differentiation of preadipocytes into adipocytes involves exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors. This cascade begins with the CCAAT/enhancer-binding protein (C/EBP)- β and C/EBP- γ , which finally induce the expression of C/EBP- α and peroxisome proliferator-activated receptor PPAR- γ

(Morrison and Farmer, 1999; Rosen et al., 2000). These transcription factors coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype, including the genes for adipocyte fatty acid binding protein (aP2), lipoprotein lipase (LPL) and leptin (Tontonoz et al., 1994). The present study aimed to study the protein and gene expression of some of these adipogenic markers in human subcutaneous adipose cells treated with either estradiol or quercetin alone or in combination. In addition to measuring PPAR- γ and C/EBP α as indicators of adipogenesis, lipid accumulation and lipogenic genes, such as SREBP1-c and its target genes like perilipin, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and adipocyte fatty acid binding protein (aP2) were used to determine the degree of differentiation and lipogenesis. Interestingly, the protein levels of lipid droplet protein, perilipin were significantly elevated for adipose cells treated with either estradiol or quercetin individually or in combination. Increased perilipin expression has been reported to suppress basal lipolysis and increase stimulated lipolysis (Souza et al., 2002). The results suggest that both estradiol and quercetin treatment enhance the lipolytic rate and may induce reductions in adipocyte size. The results of this study also reveal reduced expression of adipogenic markers that promote adipocyte lipid storage. These include lipoprotein lipase (LPL), which promotes adipocyte uptake of circulating lipids for triglyceride formation, and the lipogenic markers such as acetyl-CoA carboxylase, fatty acid binding protein and fatty acid synthase. Fatty acid metabolism is stringently controlled to balance synthesis (e.g., lipogenesis) and degradation (e.g., β -oxidation) in response to various physiological signals. Sterol regulatory element-binding proteins (SREBPs), transcription factors have been reported to be associated with lipogenesis and known to regulate the expression of ACC and FAS (Kolehmainen et al., 2001). SREBP-1c expression was markedly reduced by estradiol and quercetin treatment. Consequently, the expression of its downstream target genes such as ACC and FAS was also suppressed, suggesting that both estradiol and quercetin can effectively reduce lipid accumulation by inhibiting lipid synthesis through the SREBP-1c pathway. In parallel to the reduced expression of adipogenic markers promoting lipid storage, both estradiol and quercetin greatly reduced the expression of PPAR γ and C/EBP α . Both of these transcriptional factors play a crucial role in adipocyte differentiation. It may therefore be indicated that the inhibitory effects of estradiol and quercetin on differentiation of pre-adipocytes might be due to the inhibition of PPAR γ and C/EBP α expression.

In conclusion, the data from the present study suggest that both estradiol and quercetin can promote leanness in part by reducing adipocyte size through reduced uptake of fatty acids and

reduced lipogenesis. Quercetin, at a concentration of 50 μM , mimics estrogenic properties and acts as a phyto-estrogen. It could be used to replace estrogen, for example, in the therapy of postmenopausal estrogen deficiency. However, it should be applied with great precaution since most phyto-estrogens have antagonistic effects at different doses. More studies are therefore warranted in this regard.

Chapter 7

General Discussion, Conclusion, Limitations and Scope for Future Study.

7.1 Discussion and Conclusion

Numerous epidemiological studies report a global increase in the prevalence of obesity at alarming rates in the current modern societies (Kosti et al., 2006; Bn et al., 2007; Knai et al., 2007; Kim et al., 2012). It is a major global health problem currently affecting over 50% of the world's population. It is prevalent at all ages including babies. Both visceral as well as subcutaneous adipose tissue can accumulate fat, mainly as a result of poor nutritional habits and lack of physical activity. In turn, these are clearly responsible for increased body weight, which in turn underlies an elevated risk of diabetes and cardiovascular complications. Although it seems that individuals with higher body mass index (BMI) are prone to cardiovascular events, reasons for this connection are not entirely clear. Over the last few decades some mechanisms of diseases, especially in association with recently described dysregulation of the adipose tissue due to certain medications/drugs were discovered, shedding light on the pathophysiology of obesity-associated metabolic alterations.

On the grounds of the deregulated physiology of adipose tissue, investigation of the functions of distinct cytokines, overall redox status, transcriptional regulation and protein expression of crucial adipogenic markers may provide new insights and comprehension of the pathophysiology of T2DM and cardiovascular diseases, as well as specific targets for future therapeutic approaches. Furthermore, diagnostic tools based on knowledge about the complex interactions between metabolic and inflammatory pathways, might indicate more precisely which patients are under increased risk, prompting physicians to offer differentiated and optimized therapeutic approaches.

This study was therefore, particularly designed with the knowledge that certain medications or many drugs such as paracetamol, caffeine, rilpivirine, estradiol, β -naphthoflavone (BNF), to name a few, may contribute significantly to the process of adipogenesis, broadly termed as drug-induced metabolic alterations. The findings of the study have been presented in chapters 3-8 of this thesis. The study employed an *in vitro* adipocyte cell model in tackling the scientific problems. The main findings are summarized in figure 7.1.

The major findings of this study included the following:

1. Caffeine contains potent lipolytic effects, but no significant effect on the process of adipogenesis itself.

2. Paracetamol appears to have no significant effect on the process of lipolysis, but it can reduce triglyceride concentrations indicating that the independent use of paracetamol may contain anti-adipogenic effects and thereby prevent triglyceride formation.
3. BNF has inhibitory effects on lipolysis and attenuates any potent lipolytic effects expressed by the use of caffeine and paracetamol.
4. A combination of rilpivirine and 17 β -estradiol at dose concentrations of 10 μ M and 0.1 μ M respectively, is completely non-toxic to pre-adipocytes at most concentrations tested. During the 10-day exposure to the drugs, only estradiol alone produced a modest cytotoxic effect. No profound deleterious effect was observed on adipose tissue development or the endocrine function of adipose tissue (adipokine and cytokine release) in treated 3T3-L1 human pre-adipocytes.
5. This study reflects the lipo-atrophic effects of rilpivirine at high dosage concentrations (15 μ M) via inhibition of triglyceride accumulation and suppression of crucial markers of adipogenesis.
6. The results of this study demonstrate the potential abilities of quercetin to attenuate oxidative damage and decrease inflammatory markers. However, it failed to overcome the lipo-atrophic effects of rilpivirine.
7. Both estradiol and quercetin can promote leanness in part by reducing adipocyte size through reduced uptake of fatty acids and reduced lipogenesis. Quercetin at a dosage concentration of 50 μ M mimics estrogenic properties and acts as a phytoestrogen.

This discussion will now focus on the results present in each chapter critically comparing the present results with other studies.

Chapter 3 of this study investigated the effects of caffeine, paracetamol and β -naphthoflavone (BNF) on the differentiation of human adipose derived mesenchymal stem cells. Adipose cells were cultured in vitro using differentiation inducing media with and without the presence of different combinations of the drugs. Markers of adipogenesis were evaluated using biochemical assays including triglyceride and glycerol quantification. The results show an increase in glycerol levels for cells treated with caffeine suggesting its anti-adipogenic characteristics through the enhancement of the lipolytic process. This inhibition of adipogenesis by caffeine has been previously supported by other studies (Su et al., 2013). Paracetamol also appears to have anti-

adipogenic effects due to its apparent role in suppressing the accumulation of triglycerides. In addition, the use of β -naphthoflavone, an artificially synthesized flavanoid, in combination with caffeine and paracetamol further attenuated triglyceride accumulation as compared to the drug used without its supplementation. These results suggest important anti-adipogenic properties of β -naphthoflavone. From these findings, it is possible to conclude that caffeine, paracetamol and BNF can exert anti-adipogenic and lipolytic properties during obesity. Preliminary data obtained from this study show that these drugs may be utilized as therapeutic targets for the treatment of obesity. The results obtained in the current study corroborate similar finds by other workers (Zheng et al., 2014; Han et al., 1999; Astrup et al., 1990; Astrup et al., 1992).

Chapter 4 investigated the inflammatory status of 3T3-L1 human pre-adipocytes when treated with different concentrations of either rilpivirine or estradiol alone and in combination with β -naphthoflavone, (BNF), a potent agonist of the aryl hydrocarbon receptor. Rilpivirine is a non-nucleoside reverse transcriptase inhibitor, recently developed as a drug of choice for initial anti-retroviral (ARV) treatment of HIV-1 infection, whereas estradiol is a major component of hormonal contraceptives. Both of these drugs have effects on lipid metabolism, impairment of adipocyte differentiation and alteration of adipose tissue distribution and function. After 10 days of differentiation procedure, cells were examined for their morphology and viability. Glycerol, adiponectin, leptin, resistin and interleukin-8 (IL-8) were quantified using commercially available kits. The results show that either rilpivirine or estradiol individually or during their combination can evoke significant increases in glycerol release and a concomitant significant decrease of adiponectin from adipocytes. These effects were dose-dependent. The effects of combined treatments were much larger than individual concentration for each drug. Both drugs had little or no effect on leptin levels, except for a small decrease with 10 μ M rilpivirine alone or when combined with estradiol. In addition, both drugs evoked small increases in the release of resistin and interleukin-8 with significant values at higher doses compared to untreated adipocytes. When adipocytes were pretreated with BNF or combined with either rilpivirine or estradiol, they evoked a much larger release in glycerol and a much larger decrease in adiponectin compared to the absence of BNF. In contrast, BNF treatment had little or no effect on either leptin, resistin or IL-8 metabolism compared to the results obtained in the presence of either rilpivirine or estradiol alone or in combination. These results show that rilpivirine and estradiol either alone or when combined or pretreated with BNF can evoke marked effects on glycerol and cytokines levels from adipocytes.

From the results of this study, it is possible to conclude that a combination of rilpivirine and 17 β -estradiol is completely non-toxic to pre-adipocytes at most concentrations tested. During the 10-day exposure to the drugs, only estradiol produced a modest cytotoxic effect at the highest concentrations. No profound deleterious effect was found on either adipose tissue development or the endocrine function of adipose tissue (adipokine and cytokine release) in treated 3T3-L1 human pre-adipocytes. However, caution should be maintained considering that other transcriptional factors play a crucial role in overall alterations of lipid metabolism. BNF does not seem to significantly interact with rilpivirine and 17 β -estradiol except for the release of adiponectin. Moreover, in order to establish and elucidate a significant effect of these drugs on the adipogenic process, studies at gene expression levels and involving the markers of oxidative stress are required.

Lower atherogenic lipid profile may be among the various factors to consider when selecting the most appropriate initial anti retroviral regimen, particularly for those HIV-1 patients with a significant prior coronary heart disease risk. Including such a consideration seems warranted, since treatment of the ART-induced lipid changes with currently licensed lipid lowering agents is not without problems. The newer classes of ART, including rilpivirine, are still in the early phase of treatment, and studies determining the effects on metabolic indices and body composition are limited. Data to date suggest fewer metabolic consequences of newer ART compared with older regimens. However, potential metabolic complications have been identified early in the development of these agents which warrants awareness and merits further long-term studies.

Chapter 5 of this study provides a detailed assessment of the action of rilpivirine on human adipocytes. The study concludes that rilpivirine exerts lipo-atrophic effects on adipose cells by impairing triglyceride accumulation, increasing inflammation, repressing anti-oxidant enzymes and inhibiting the expression of genes controlling adipogenesis (PPAR γ , C/EBP α , SREBP-1c, fatty acid synthase and aP2). The results confirm previous findings of mitochondrial DNA depletion, inflammation, and disturbances in adipogenesis in lipoatrophic fat in adipose cells on treatment with other antiretroviral drugs. The effects of rilpivirine were qualitatively similar to those of efavirenz reported previously in closely related human cell culture models (Gallego-Escuredo et al., 2010; Díaz-Delfín et al., 2011, 2012). An analysis of the effects of efavirenz and elvitegravir at similar concentrations, especially in the range between 0.5 and 2 mM, indicated that efavirenz exerted similar but somewhat stronger deleterious effects on adipogenesis (e.g. adipose morphology, PPAR γ and GLUT4 expression) than elvitegravir.

Administration of ART can greatly reduce HIV-related inflammation. This inflammation is normally exerted in the form of altered cytokine release, adipocyte dysfunction, metabolic disturbances and oxidative stress are part of the body's effort to fight the virus. Unfortunately, medications do not completely eliminate the inflammation. The immune system continues to be activated and low-level inflammation persists even when the viral load is undetectable. In the recent years, research findings on the benefits of anti-oxidant foods and supplements like ginger, turmeric, cherries, onions, garlic, vitamin c and E, foods rich in omega -3 fatty acids etc. have been employed in countering both oxidative stress and inflammation and to reduce the adverse side effects of ART. Combining the presently available natural flavanoids with anti-inflammatory and anti oxidative properties and help to overcome the side effects incurred due to the anti-retroviral therapy. The present study also focused on the combinatory effects of rilpivirine and quercetin. The results conclude that quercetin was able to decrease inflammation and restore the levels of anti-oxidant enzymes but failed to overcome the lipotrophic effects of rilpivirine (Asrer et al., 2005; Murata et al., 2014; Saravanan et al., 2015).

As more research studies are trying to progress towards a cure to prevent HIV, novel ART are actively being developed, which may be able to control viral suppression and simultaneously inhibit critical inflammatory and atherogenic pathways. Taken together, the positive effects of ART on viral suppression far outweigh negative effects on metabolic indices, but future studies should focus on the development of even more potent novel strategies with optimal immunomodulatory and metabolic profiles. So far, the data from the present study seem to suggest that the use of antioxidative dietary flavonoids such as quercetin may not be seen as a promising approach in the prevention of adverse metabolic syndrome induced by ART.

Estrogens are crucial hormonal regulators of systemic energy homeostasis in both sexes, and there is increasing evidence that E2 treatment exhibits a protective effect in humans and rodents against the development of obesity-related metabolic disorders such as type 2 diabetes via the regulation of adipose tissue metabolism. Quercetin is a phytoestrogen that exerts both in vitro agonistic and antagonistic activities on estrogen receptors. Chapter 6 of this study was designed to elucidate the role of 17 β -estradiol on human subcutaneous cells in vitro either individually or in combination with quercetin. Despite data demonstrating that estrogen functions as a negative regulator of adipose development, it has also been suggested that E2 can stimulate adipocyte proliferation and differentiation. It is further reported that E2 can enhance the proliferative capacities of subcutaneous preadipocytes from female rats, but not

from male rats (Dieudonne et al., 2000). In human preadipocytes, E2 stimulates cell proliferation in a dose-dependent manner with a higher response to E2 in women than in men (Anderson et al., 2001). A more recent report showed that estrogen sulfotransferase (EST), a cytoplasmic enzyme that inactivates estrogens through sulfation of their hydroxyl group, inhibited adipocyte differentiation in 3T3-L1 cells, and overexpression of EST in the adipose tissue of female mice resulted in a smaller adipocyte size (Wada et al., 2011). Taken together, estrogens may regulate adipose development with depot- and gender-specific differences, but their effects in the context of adipose tissue function remain unclear. In the present study both estradiol and quercetin either individually and in combination attenuated lipid accumulation as determined by adipore staining and quantitative triglyceride measurement in a dose-dependent manner. Number of previously conducted studies have shown that estrogen may contribute to the prevention and/or improvement of obesity-related metabolic syndromes by altering the production of adipocytokines in rodents, monkeys and humans (Hong et al., 2007; Kafkas et al., 2012; Shaw et al., 2013). One of the adipocytokines that shows a strong correlation with estrogen is leptin. Data from the present study show increased leptin levels when adipose cells were treated with estradiol in comparison to non-treated cells. These results are in parallel with other studies showing increased levels of leptin in adipose tissue when treated with estrogen (Shimizu et al., 1997; Fungfuang et al., 2013). Leptin levels remained unaltered when adipose cells were treated with quercetin alone or in combination with estradiol. In the present study, 17 β -estradiol dramatically suppressed the level of adiponectin. This decrease in adiponectin concentration seems to be associated with insulin resistance and other related disorders (Kowalska et al., 2008). Treatment of adipose cells with quercetin alone or in combination with estradiol significantly increased adiponectin levels demonstrating the anti-inflammatory properties of quercetin. The present study showed copiously elevated resistin concentration levels when adipose cells were treated with estradiol. This increase in resistin concentration may be attributed to the expression of CEBP α which acts as a transcriptional activator of resistin. This implication is also indirectly supported by the findings that a functional C/EBP-binding site was found in the proximal resistin promoter of both murine and human adipocytes to be necessary for the stimulation of transcription from resistin promoter (Hartman et al., 2002; Song et al., 2002; Seo et al., 2003) and that endogenous C/EBP was bound to the resistin promoter in adipocytes in association with p300 and CREB-binding protein, which are viewed as co-activators involved in ER activation (Nilsson et al., 2001). Adipose cells treated with quercetin alone or in combination with estradiol showed decreased resistin concentration in adipose cells.

Although not much studied, IL-8 has been reported to be produced and released from human adipose tissue in regulated manner (Bruun et al., 2001; Gerhardt et al., 2001). Besides the participation in inflammatory processes, its involvement has been suggested in the development of obesity-associated insulin resistance (Kern et al., 2001), atherosclerosis and cardiovascular disease (Boisvert et al., 2000; Schieffer et al., 2000). The present study showed significant reduction in IL-8 levels when adipose cells were treated with estradiol or quercetin alone and in combination, suggesting the protective effects of these two drugs on the development of metabolic syndrome. In humans, oxidative stress is thought to be involved in the pathogenesis and development of metabolic syndrome. The results of the present study on human adipocytes *in vitro* show that both estradiol as well quercetin decreased the nitric oxide levels and increased the anti-oxidant enzyme activities suggesting a protective role of these drugs in adipose tissue.

The differentiation of preadipocytes into adipocytes is regulated by a complex network of transcription factors. In the present study, both estradiol and quercetin treatment markedly reduced the expression levels of C/EBP α and PPAR γ compared with those in differentiated control cells. Since both of these markers are master regulators of adipogenesis; their maintenance is critical to the progression of the final stages of adipocyte differentiation. Thus, these results indicate that both estradiol, as well as quercetin can significantly reduce lipid accumulation by down-regulation of these adipogenic markers. Experimental data also show a dramatic down-regulation of SREBP1-c expression. Because the mature form of SREBP1-c is known to promote lipogenic gene expression (Foufelle et al., 2002), its decreased levels may contribute significantly to impaired lipogenesis in estradiol and quercetin-treated adipose cells. The data in this study also show a decrease in the expression of its target genes such as FAS, FABP2 and ACC suggesting anti-adipogenic as well as anti-lipogenic action of estradiol and quercetin in adipose cells.

Conclusion:

The main conclusions from this study include the following:-

1. Caffeine, paracetamol and BNF can exert anti-adipogenic and lipolytic properties during obesity.

2. A combination of rilpivirine and 17 β -estradiol is completely non-toxic to pre-adipocytes at most concentrations tested. During the 10-day exposure to the drugs, only estradiol produced a modest cytotoxic effect at the highest concentrations.
3. The results show that rilpivirine and estradiol either alone or when combined or pretreated with BNF can evoke marked effects on glycerol and cytokines levels from adipocytes. The present study also focused on the combinatory effects of rilpivirine and quercetin and from the results it is possible to conclude that quercetin was able to decrease inflammation and restore the levels of anti-oxidant enzymes but failed to overcome the lipo-atrophic effects of rilpivirine.
4. The results of this study show a decrease in the expression of its target genes such as FAS, FABP2 and ACC suggesting anti-adipogenic as well as anti-lipogenic action of estradiol and quercetin in adipose cells.
5. The main conclusion from this study is that some antiviral drugs can induce obesity, but if they are used in combination with some plant-based antioxidant drugs then their adverse effects can be avoided.

7.2 Limitations of the study

Some of the limitations to the present study included:-

1. In order to establish and elucidate a significant effect of caffeine, paracetamol and BNF on the adipogenic process, further experiments at gene expression levels and involving the markers of oxidative stress should have been done.
2. Apart from quercetin, other natural flavonoids should have been tested to study potential interactions between ARVs and plant-derived products.
3. Combinations of ARVs, PPAR γ agonists/antagonists were not used and lipolysis was not investigated; thus it is important to address these points in future studies to mimic current use of fixed dose drug combinations among patients, to determine whether adipocyte dysfunction is mediated via PPAR- γ and to determine the contribution of lipid mobilizing factors and lipolysis to the pathogenesis of HALS.
4. Most phytoestrogens have antagonistic effects at different doses. The present study tested quercetin at a dose concentration of 50 μ M only. More concentrations must be tried.

7.3 Scope for Future Studies

The results from the present study have opened up new avenues and opportunities in the field of obesity research. Some of these are briefly described below:

1. Many natural products and bioactive compounds such as possess remarkable pharmacological activities against obesity and hold great promise for development into potential anti-obesity drugs, current knowledge of their exact mechanism of actions remained to be fully elucidated. For instance, further studies are needed to help us understand their effect on the epigenetic regulation of obesigenic genes.
2. Data from present obesity research studies as well as other disorders with a complex aetiologies support that alleles in a number of genes can contribute to obesity. However, the extent to which these common alleles exert their impact or rare alleles with more measurable effects on a given susceptibility background is still uncertain. Different ethnicity of analyzed cohorts and different ascertainment criteria, as well as different analyzed obesity phenotypes, make it more difficult to compare multiple allelic association studies of the same obesity candidate gene. This may partially explain the difficulty to confirm reported association between specific gene alleles and obesity. Defining gene alleles that are associated with obesity will contribute to our understanding of the underlying mechanisms behind its development and potentially provide therapeutic targets.
3. The chronic and co-morbid nature of HIV infection has necessitated the use of multiple drugs including herbs to overcome its adverse side effects with a possible increase in herb–drug interaction cases. The present study shows the effect of quercetin to overcome the lipodystrophic effects caused due to rilpivirine treatment. The therapeutic potential of other flavanoids and supplements must be explored. However, one must bear in mind that most of the ART drugs are metabolized by cytochrome P450 (CYP) enzymes similar to flavanoids. Concurrent use of herbal drug may result in an interaction that may significantly inhibit or induce these enzymes, potentially resulting in toxicity or therapeutic failure, respectively. Further experiments are required in this area of herbal drug combination therapy.
4. The recent demonstration of ubiquitous presence of xenobiotic molecules in living organisms with endocrine disrupting properties with the ability to change estrogen signaling also deserves attention and may provide clues to the epidemic growth of obesity-related metabolic complications. Even so, there is general agreement that estrogens can improve

inflammation related to endocrine-disrupting chemicals which in turn may mediate their actions on metabolism. Although it seems evident that it acts indirectly through metabolic amelioration, the direct regulation of inflammation pathways is also documented. However, much work still needs to be performed in this respect to allow broadening the knowledge and understanding on estrogens' mechanisms of action and establishing the rationale for the development and use of estrogen signaling modulation as a therapeutic tool for metabolic improvement.

Hopefully, this thesis will provide novel ideas for development of treatment strategies for this 21st century "obesity bomb" and its related disorders.

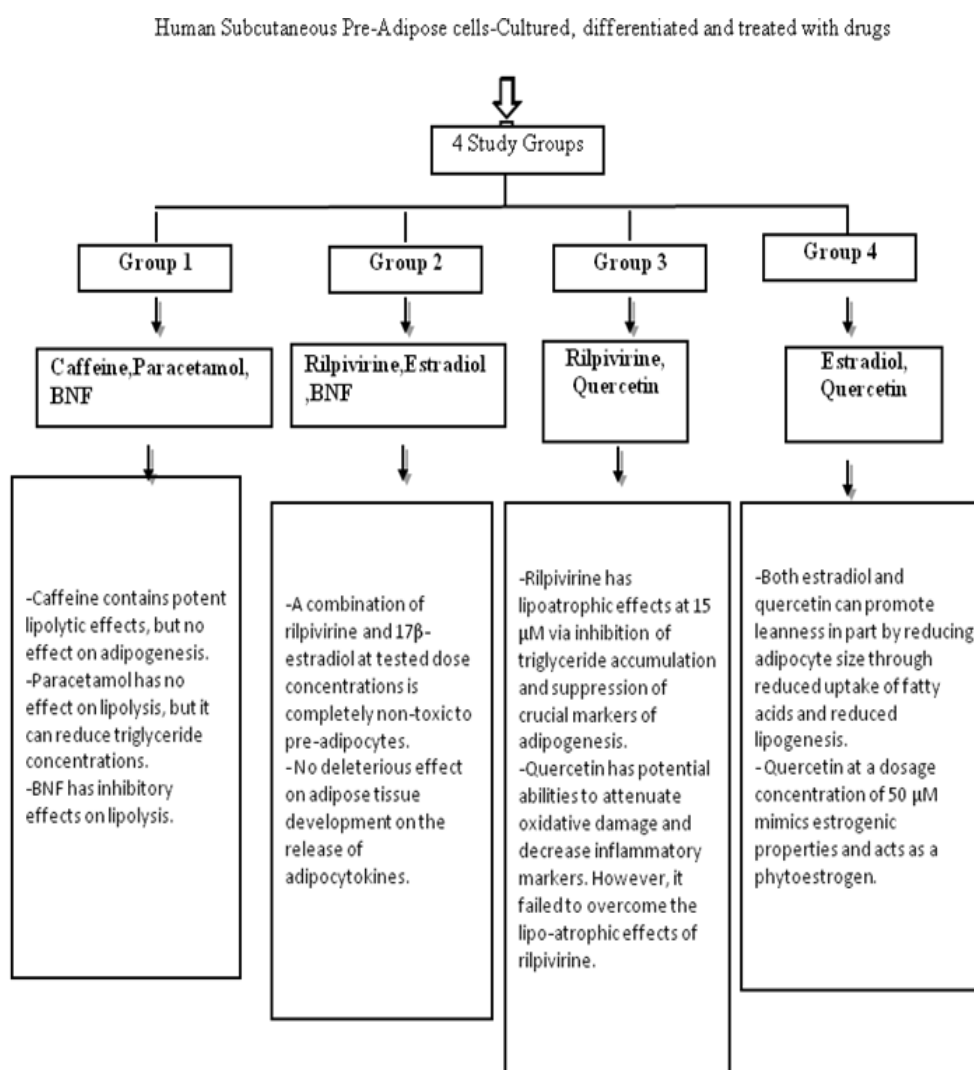


Figure 7.1: Flow diagram highlighting the main findings from the present study.

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Appendices

Chapter 1 (Appendix)

Fabp-2 and Ppar- γ genes as risk factors for dyslipidemia in type 2 diabetes mellitus in residents of United Arab Emirates.

Introduction: Diabetes mellitus (DM) is one of the most prevalent chronic disorders worldwide and poses a great health concern due to its associated risks. Its elevated prevalence, morbidity and mortality rates and the social and economic repercussions of its chronic long-term complications compromise both the quality of life and productivity of those affected, making it a serious public health problem (Gulliford et al., 2009). According to the World Health Organization, 450 million people worldwide suffer from diabetes and the prevalence is expected to rise to 650 million by 2030 (Mihardja et al., 2014). Statistical figures from the International Diabetes Federation (IDF) revealed that in 2017, 17.3 % of the UAE population, between the ages of 20 and 79 years of age had type 2 diabetes (T2DM). Currently, the percentage has increased to around 20 %. There are over 1 million people living with diabetes in the United Arab Emirates (UAE) and another 3 million with pre-diabetes, placing the country 15th worldwide for age-adjusted comparative prevalence. It is important to note that diabetes is a regional affliction with Saudi Arabia, Bahrain, Kuwait and Qatar all featuring in the top twenty countries in terms of prevalence worldwide (International Diabetes Federation, 2017). Current trend also indicates that the prevalence of diabetes in the UAE is rising at a faster rate than both the Middle East and Northern Emirates (MENA) region and the rest of the world. Rapid economic growth, sedentary lifestyle and unhealthy diets characteristic of the UAE are all risk factors, leading to the number of people with diabetes expecting to double to 2.2 million by 2040. This number not only symbolizes colossal societal costs in terms of morbidity and mortality, but also in terms of healthcare system and productivity. T2DM presents a crucial health problem due to its constantly elevating rate of morbidity and mortality, which is related to metabolic disorders and cardiovascular risks, comprising of insulin resistance, obesity, dyslipidemia, hypertension, atherosclerosis, and endothelial dysfunction. Dyslipidemia, one of the major risks arising from diabetes, is defined as the abnormal change in levels of plasma cholesterol, triglycerides (TGs), or both, or a low high density lipoprotein level that results in the development of atherosclerosis. The characteristic features of dyslipidemia demonstrate decreased high-density lipoprotein cholesterol (HDL-C) levels, raised low-density lipoprotein cholesterol (LDL-C), and elevated triglyceride levels. Insulin resistance is known to introduce this triad of low HDL, high LDL and triglyceride levels (Reaven, 1988; Hoenig et al., 2010). The incidence of lipid abnormality is very common in patients with T2DM than in general population. This is because in diabetes, insulin resistance or deficiency affects key enzymes and pathways in lipid metabolism. These abnormalities arise from increased secretion of VLDL (very-low-density lipoprotein) particles due

to elevated concentrations of free fatty acids and glucose (Eckel, 2014; Filippatas et al., 2017; Verges, 2015; Ginsberg, 1987)

Data from a study, conducted on Argentina population by the National University of San Luis in 2014 showed an association of FABP2 (Fatty Acid Binding Protein-2) and PPAR- γ (Peroxisome proliferator-activated receptor) genes with dyslipidemia in diabetic patients. It was concluded that in T2DM, the additive effects of the Threonine-Proline (Thr-Pro) Haplotypes of FABP2 and PPAR- γ genes altered the lipid metabolism (Conlara et al., 2016). FABP-2 gene has been proposed to be a suitable candidate gene for diabetes since this gene is involved in the absorption of fatty acids across intestinal mucosa. The risk of lipid metabolic disorders occurrence in individuals carrying mutational FABP2 gene (Ala54/Thr54, Thr54/Thr54) has been reported to be higher than those carrying wild type homozygote (Ala54/Ala54) when they were exposed to the same environment (Liu et al., 2015). Peroxisome Proliferator Activated Receptor (PPAR- γ) is a transcription factor protein with chief role in adipogenesis and insulin sensitization. It is highly expressed in adipose tissue, where it plays an indispensable role in the regulation of adipocyte differentiation, lipid storage, glucose metabolism and the transcriptional regulation of a number of genes involved in these metabolic processes (Semple et al., 2006; Azhar, 2010). Frequent mutations in the PPAR- γ gene have been reported to be associated with obesity and diabetes-related phenotypes. Previous studies have confirmed that the common structural polymorphism with a proline to alanine substitution affects the function (Deeb et al., 1998). In comparison with the Pro allele, the Ala allele is also associated with reduced activity of PPAR- γ . Several studies conducted on PPAR- γ polymorphisms have been extensively investigated by different researchers have suggested its relation with obesity and T2DM and is considered to be one of the best replicated genetic risk factors of common T2DM (Altshuler et al., 2000; Lohmueller et al., 2003; Tönjes et al., 2006). Basically, the Ala allele variant has a reduced activity of PPAR- γ compared to the Pro allele; so this mutation causes reduced insulin sensitivity leading to the development of T2DM.

Aims: Considering the importance of allelic polymorphisms in these genes in the development of obesity, the present study was designed to evaluate the association between single nucleotide polymorphisms and haplo-types of the FABP2 and PPAR γ and lipid profile in the residents of United Arab Emirates.

Materials and methods

Blood Sampling

Blood samples were obtained from patients with overnight fasting for a minimum of 12 hours by trained laboratory personal and stored at 4 °C. A volume of 3 ml of blood was collected in tubes containing 0.1 % EDTA.

Biochemical Measurements

Fasting Plasma Glucose

Plasma glucose was measured by strip-based assay using glucometer from AccucheckTM.

Quantification of total Cholesterol

Total cholesterol was measured using the Total Cholesterol Quantification Kit from Sigma (Cat. #: MAK043) as per the manufacturer's protocol. The kit was shipped on wet ice and stored at -20°C upon receipt. For the assay procedure, all samples and standards were run in duplicate. In order to perform the colorimetric detection, a volume of 20 mL of the 2 mg/mL Cholesterol Standard solution was diluted with 140 mL of the Cholesterol Assay Buffer to prepare a 0.25 mg/mL standard solution. A volume of 0, 4, 8, 12, 16, and 20 mL of the 0.25 mg/mL Cholesterol Standard solution was added into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 mg/well standards. This was followed by addition of Cholesterol Assay Buffer to each well to bring the final volume to 50 mL. Serum samples (0.5–5 mL/well) were added directly to each of the sample wells. For the assay reaction, reaction mixes were prepared according to the scheme in table 1.

Table 1: Table showing scheme for preparing the reaction mixes for cholesterol assay

Reagents	Total cholesterol and standards	Free cholesterol
Cholesterol Assay buffer	44 µl	46 µl
Cholesterol Probe	2 µl	2 µl
Cholesterol Enzyme Mix	2 µl	2 µl
Cholesterol Esterase	2 µl	-

A volume of 50 mL of the appropriate reaction mix was added to each of the wells followed by incubation for 60 minutes at 37°C. The plate was protected from light and absorbance was measured at 570 nm. The amount of cholesterol present in the samples was determined from the standard curve. Concentration of Cholesterol was calculated using the following equation:

$$S_a/S_v = C$$

Where,

S_a = Amount of cholesterol in unknown sample (mg) from standard curve

S_v = Sample volume (mL) added into the wells

C = Concentration of cholesterol in sample

Quantitative measurement of High-Density Lipoprotein and Low-Density Lipoprotein

Abcam's HDL and LDL/VLDL Cholesterol Quantification Kit (Cat. # ab65390) was used as per the manufacturer's protocol. The kit was stored at -20°C in the dark immediately upon receipt. The kit components included cholesterol assay buffer, 2x LDL/VLDL precipitation buffer, cholesterol standard (2 µg/µL), cholesterol probe, cholesterol esterase and enzyme mix. Lyophilized cholesterol esterase and enzyme mix were dissolved in 220 µL of cholesterol assay buffer and kept on ice during the assay. A volume of 200 µL of 0.25 µg/µL cholesterol standard was prepared by diluting 25 µL of the provided cholesterol standard (2 µg/µL solution) with 175 µL of cholesterol assay buffer. This was further used to prepare working cholesterol standards as outlined in table 2 below.

Table 2: Preparation of cholesterol standards using serial dilution

Standard #	Cholesterol 0.25 µg/µL Standard (µL)	Assay Buffer (µL)	Final Volume Standard in well (µL)	End amount cholesterol in well (µg/well)
1	0	150	50	0
2	12	138	50	1
3	24	126	50	2

4	36	114	50	3
5	48	102	50	4
6	60	90	50	5

For the quantification of total cholesterol, serum samples were used directly. For the separation of HDL and LDL/VLDL fraction, a volume of 100 μ L of sample was mixed with a volume of 100 μ L of 2x precipitation buffer in micro-centrifuge tubes and incubated for 10 minutes at room temperature. This was followed by centrifugation at 5000 rpm for 10 minutes. The supernatant was then transferred to a new tube. This was the HDL fraction. The remaining precipitate was re-suspended in 200 μ L of PBS. This was the LDL/VLDL fraction.

For the assay procedure, all materials and prepared reagents were gently agitated and equilibrated to room temperature prior to use. All standards, controls and samples were assayed in duplicate. For setting up the reaction wells, a total volume of 50 μ L of standard dilutions as well as samples were loaded onto the plate. This was followed by the addition of 50 μ L of total cholesterol reaction mix (44 μ L of cholesterol assay buffer + 2 μ L of cholesterol probe + 2 μ L of enzyme mix and 2 μ L of cholesterol esterase) and 50 μ L of free cholesterol reaction mix (46 μ L of cholesterol assay buffer + 2 μ L of cholesterol probe + 2 μ L of the enzyme mix) into each of the wells. Enough reagents for the number of assays to be performed were prepared and a master mix was prepared to ensure consistency. All the wells were mixed thoroughly and incubated at 37°C for 60 minutes protected from light. Absorbance was taken at OD 570 nm using a micro-plate reader. For data analysis, corrected absorbances were calculated by subtracting mean value of blank (standard # 1) from all standards and samples. Corrected values for each standard were plotted as a function of the final concentration of triglyceride. Corrected sample OD was applied to the standard curve to get cholesterol (A) amount in the sample wells. Concentration of cholesterol in (μ g/ μ L) in the test samples was calculated using the following equation:

$$\text{Cholesterol concentration} = (A/V \cdot D) \cdot DF$$

Where:

A= Amount of cholesterol in the sample well calculated from the standard curve (μ g)

V= Sample volume added in the sample wells (μL)

D= Sample dilution factor: For total cholesterol = 1; for HDL and LDL/VLDL fractions =2

DF = Additional dilution factor if sample has been diluted further to fit within the standard curve range.

DNA Extraction and Restriction Fragment Length Polymorphism for Genotyping Analysis

DNA was extracted from packed blood cells using the QiAmp Mini Kit™ from Qiagen (Cat. # 51104) following the manufacture's protocol. The kit components included QIAamp Mini Spin Columns, 2 ml collection tubes, Buffer AL, Buffer ATL, Buffer AW1 (concentrate), Buffer AW2 (concentrate), Buffer AE, QIAGEN® Protease, Protease Solvent and Proteinase K. Figure 1 showed a simplified procedure for the extraction of DNA using spin column method.

QIAamp Spin Procedure

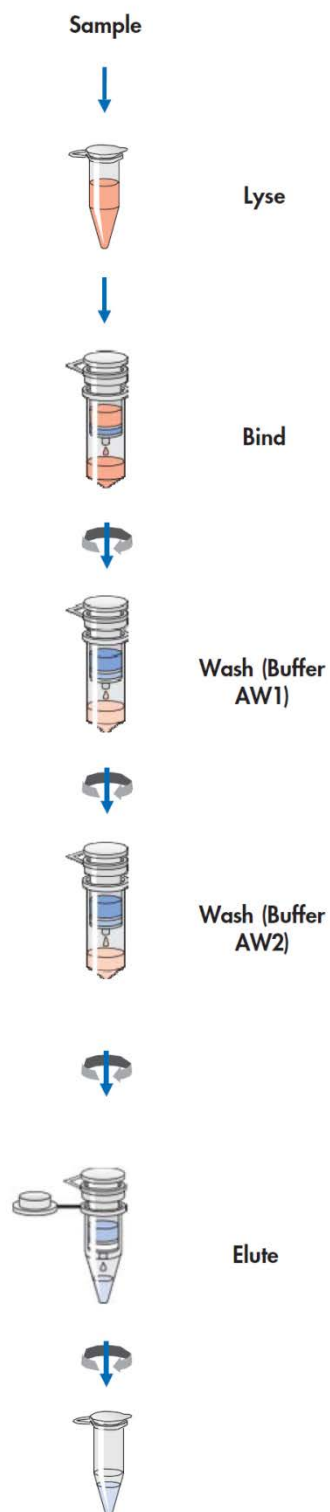


Figure 1: Diagrammatic scheme for the extraction of DNA using Spin column method.

PCR amplifications and genotype determinations were conducted as follows:

FABP-2 (Ala54Thr): Ala54Thr (G/A) in exon 2 of FABP2 (rs1799883). DNA from the samples were amplified using a total volume of 20 µl containing 100 ng of genomic DNA, 12 pmol of each primer and 1 unit of DNA polymerase. These reactions were performed in a 2X Master Mix containing 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), and 200 pmol/L of each deoxynucleotide triphosphate. The primers were as follows: forward primer: 5'ACAGGTGTTAATATAGTGAAAAG3' and reverse primer: 5'TACCCTGAGTTCAGTTCGTC3'. The template DNA was denatured for 3 minutes at 95°C before undergoing 30 cycles of amplification. Each amplification cycle included: Denaturation for 30 seconds at 95°C, primer annealing for 30 seconds at 55°C, and extension for 45 seconds at 72 °C; followed by a final extension at 72 °C for 3 minutes.

For Restriction Fragment Length Polymorphism (RFLP) analysis, 1 µl of PCR product was incubated with 1 µl of *CfoI* enzyme (10 U/µl, in a final volume of 20 µl for 1 hour at 37 °C. The products were run on a 2% non-denaturing Polyacrylamide gel electrophoresis (PAGE) for 30 minutes at 100 V. Bands was observed after ethidium bromide staining and ultraviolet (UV) light exposure. Visualization of two DNA fragments of the *CfoI* treated amplicon at 99 and 81 bp will indicate a G allele (Ala54—presence of the restriction site), whereas an intact 180 bp will indicate an A allele (Thr54—absence of the restriction site).

PPAR-γ (Pro12Ala). The PPAR-γ polymorphism (rs1801282) Pro12Ala (C/G) was analyzed by Tetra Primer AMRS-PCR. Two pairs of primers were used, one which amplifies a fragment of 553 bp, common to both alleles (outer primers: forward 5'AGACAGTGTGGCAATATTTCCCTGTAA3' and reverse 5'GGTTCTGAACATGTTTTAAATGAACGC3' and another pair specific for the SNP (inner primers): forward 5'GAAACTCTGGGAGATTCTCCTATTGTCC3' for the C allele (Pro12) and reverse 5'GTATCAGTGAAGGAATCGC TTTCAGC3' for the G allele (Ala12). Nucleotide sequence and single nucleotide polymorphism (SNP) details were obtained from National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>). The primers were synthesized by Accuvis Bio (Abu Dhabi, UAE). Each PCR reaction was carried out in a total volume of 20 µL, containing 200 ng of template DNA, 1 pmol of each inner primer, and 1 pmol of each outer primer (1:1 ratio of outer to inner primer). These reactions were performed in 2X Master Mix containing 200 µM dNTPs, 2.5 mM MgCl₂, and 1 unit of DNA polymerase. The template DNA was denatured for 3 minutes at 95 °C before undergoing 35 cycles of denaturation for 1 minute at 95°C, primer annealing for 1 minute at 58.9°C, and extension for 1

minute at 72°C, and final extension at 72°C for 5 minutes. The resultant products were obtained after PCR were separated by electrophoresis on 1 % agarose gel containing ethidium bromide. The image was visualized and photographed under UV trans-illuminator. This resulted in 3 DNA fragments: one of 553 bp, one of 253 bp for the C allele (Pro12) and one of 354 bp for the G allele (Ala12).

Randomly selected 20% of samples were re-genotyped for cross validating initial genotypes. In case of unclear genotyping results, the samples were repeated again in duplicates until clear genotype was available. Unclear genotyping results, even after repetition, were excluded from the study. No genotyping error was observed during cross validation.

Results

Biochemical analysis

Table 3: Table showing the effect of dyslipidemia on the biochemical characteristics

Characteristics	Dyslipidemia (present/absent)	N	Mean \pm SEM
Low density lipoprotein (LDL)	Present	44	145.02 \pm 15.46
	Absent	20	106.95 \pm 6.37
High density lipoprotein (HDL)	Present	44	33.98 \pm 1.44
	Absent	20	49.35 \pm 2.66
Triglycerides (TG)	Present	44	170.30 \pm 15.80
	Absent	20	108.75 \pm 11.01
Total cholesterol (TC)	Present	44	195.86 \pm 8.43
	Absent	20	177.95 \pm 7.88
Fasting plasma glucose (FPG)	Present	44	129.14 \pm 6.73
	Absent	20	103.10 \pm 4.09

Table 4: The effect of gender on the biochemical characteristics

Characteristics	Dyslipidemia (present/absent)	N	Mean \pm SEM

Low density lipoprotein (LDL)	Male	38	132.55 ± 17.00
	Female	26	133.96 ± 11.19
High density lipoprotein (HDL)	Male	38	38.74 ± 1.77
	Female	26	38.85 ± 2.90
Triglycerides (TG)	Male	38	168.37 ± 16.75
	Female	26	125.77 ± 15.11
Total cholesterol (TC)	Male	38	184.39 ± 7.82
	Female	26	198.85 ± 10.62
Fasting plasma glucose (FPG)	Male	38	122.47 ± 7.66
	Female	26	118.85 ± 5.37

Table 5: The effect of nationality (UAE Local and Expatriates) on the biochemical characteristics. Data are expressed as Mean ± SEM; *p<0.05.

Characteristic	Dyslipidemia(present/absent)	N	Mean ± SEM
Low density lipoprotein (LDL)	UAE Local	20	129.65 ± 10.06
	Expatriates	44	134.71 ± 15.40
High density lipoprotein (HDL)	UAE Local	20	34.95 ± 1.85
	Expatriates	44	40.52 ± 2.07*
Triglycerides (TG)	UAE Local	20	139.60 ± 14.46
	Expatriates	44	156.27 ± 16.05
Total cholesterol (TC)	UAE Local	20	196.40 ± 11.72*
	Expatriates	44	187.48 ± 7.60
Fasting plasma glucose (FPG)	UAE Local	20	114.05 ± 5.44
	Expatriates	44	124.16 ± 6.85*

The results for the effect of dyslipidemia on the different biochemical analysis as illustrated in tables 3-5 show that dyslipidemia has significant (p<0.05) effect on high density lipoproteins,

triglycerides and fasting plasma glucose compared to subjects who did not have dyslipidemia. No significant ($p>0.05$) effect was observed for the low-density lipoproteins and total cholesterol. Statistical analysis for each of gender and nationality also showed no significant ($p>0.05$) effect on the biochemical characteristics as illustrated in tables 4 and 5, respectively.

Frequencies for genotyping polymorphisms

Table 6: Description of the Selected SNPs for FABP-2 and PPAR- γ Genes

SNP ID	Chromosome	Position	Exon/Intron	Substitution	Functional consequence	MAF ^a
FABP-2	4	120241902	Exon 2	G/A	Missense	0.30
PPAR- γ	3	12393125	Exon A1	C/G	Missense	0.25

MAF^a: in the total group of this study. MAF, Minor Allele Frequency; SNP, Single Nucleotide Polymorphism

Table 6 provides detailed information of the selected SNPs, including their features, allelic variants, and the minor allele frequency. The frequencies of the Ala54Ala, Ala54Thr, and Thr54Thr genotypes in the whole population of the FABP-2 polymorphism were 34.37%, 46.87%, and 18.75, respectively, while the frequencies of Pro12Pro, Pro12Ala, and Ala12Ala genotypes of the PPAR- γ polymorphism were 70.31%, 20.31%, and 9.37%, respectively. The distribution and the allele frequency of the 2 polymorphisms followed Hardy-Weinberg equilibrium.

Inheritance Model

Table 7: Frequencies of FABP-2 genotypes according to T2DM

Models ^a	Genotypes ^b	Control Subjects n (%) ^c	Diabetic Subjects n (%) ^c
Co-dominant	AlaAla	7(63.63)	15(28.30)
	AlaThr	1(9.09)	29(54.71)
	ThrThr	3(27.27)	9(16.98)
Dominant	AlaAla	7(63.63)	15(28.30)
	AlaThr/ThrThr	4(36.36)	38(71.69)
Recessive	AlaAla/ThrThr	8(72.72)	44(83.01)
	ThrThr	3(27.27)	9(16.98)

Over-dominant	AlaAla/ThrThr	10(90.90)	24(45.28)
	AlaThr	1(9.09)	29(54.71)

^aInheritance Models; ^bGenotypes and their grouping for the FABP-2 polymorphism (rs1799883);

^cGenotype Frequency expressed as number of individuals. N-value in parenthesis indicates percentage.

The genotypes of FABP-2 were associated with T2DM according to all possible genetic models (Codominant, Dominant, Recessive, and Over-dominant). In addition, there was no association of FABP-2 Ala54Thr polymorphism found with T2DM according to any genetic model used (Table 7). For the PPAR-γ Pro12Ala polymorphism, no association found with T2DM according to any genetic model used (SeeTable 8).

Table 8: Frequencies of PPAR-γ genotypes according to T2DM

Model ^a	Genotype ^b	Control Subject n (%) ^c	Diabetic Subject n (%) ^c
Co-dominant	ProPro	5(45.45)	40(75.47)
	ProAla	1(9.09)	12(22.64)
	AlaAla	5(45.45)	1(1.88)
Dominant	ProPro	5(45.45)	40(75.47)
	ProAla/AlaAla	6(54.54)	13(24.52)
Recessive	PrpPro/ProAla	6(54.54)	52(97.11)
	AlaAla	5(45.45)	1(1.88)
Over-dominant	ProPro/AlaAla	10(90.9)	41(77.35)
	ProAla	1(9.09)	12(22.64)

^aInheritance Models; ^bGenotypes and their grouping for the PPAR-γ polymorphism (rs1801282);

^cGenotype Frequency expressed as number of individuals. N-value in parenthesis indicates percentage

Table 9: Genotypes and allele frequencies for the FABP-2 and PPAR-γ polymorphisms in control and T2DM

SNP	Allele	Control	Diabetic	Genotype	Control N (%) ^b	Diabetic N (%) ^b
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		Fa	Fa			
rs1799883	Ala	0.68	0.56	AlaAla	7(63.63)	15(28.30)
FABP-2	Thr	0.32	0.44	AlaThr	1(9.09)	29(54.71)
				ThrThr	3(27.27)	9(16.98)
rs1801282	Pro	0.5	0.87	ProPro	5(45.5)	40(75.5)
PPAR-γ	Ala	0.5	0.13	ProAla	1(.09)	12(22.6)
				AlaAla	5(45.5)	1(1.9)

Table 9 shows the frequency of the rare Thr54 allele of the FABP-2 polymorphism in control (0.32) and this value was not different from the frequency in T2DM (0.44). The frequency of the rare Ala12 allele of the PPAR-γ polymorphism in control was however different from the frequency in T2DM (0.5 and 0.13, respectively). Since the homozygous genotype had very low frequencies, the Thr54Thr of the FAPB2 and the Ala12Ala of the PPARγ were examined in the same category as the corresponding heterozygote genotype, i.e non Ala54Ala (Thr carriers) and non Pro12Pro (Ala carriers), respectively.

Table 10: Anthropometric and clinical characteristics by the FABP-2 polymorphism in controls and T2DM

Clinical Characteristics (mg/dL)	Control (n=11) Mean ± SD		Diabetic (n=53) Mean ± SD	
	Ala54Ala	Non Ala54Ala	Ala54Ala	Non Ala54Ala
Fasting plasma glucose (FPG)	94.57 ± 11.85	107.25 ± 4.64	109 ± 18.70	132.05 ± 47.43
Triglycerides (TG)	11.14 ± 58.87	175 ± 67.05	108.13 ± 42.69	172.28 ± 110.79
Total cholesterol (TC)	193.57 ± 39.52	193 ± 58.73	178.2 ± 36.85	194.13 ± 57.15
High density lipoprotein (HDL)	55.91 ± 16.78	41.75 ± 19.82	43.74 ± 8.44	33.46 ± 8.14

Low density lipoprotein (LDL)	118.62 ± 25.80	124.75 ± 51.51	111.64 ± 52.31	145.60 ± 109.01
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Table 10 shows the anthropometric and clinical characteristics of controls and T2DM for the Ala54Thr polymorphism (rs1799883) of the FABP-2 gene. The differences between control patients with Ala54Ala and non-Ala54Ala genotypes were associated with LDL-c, whereas in T2DM the presence of the non-Ala54Ala genotype was associated with an increase in triglycerides and LDL-c compared with the Ala54Ala homozygotes.

Table 11: Anthropometric and Clinical characteristics by the PPAR-γ polymorphism in controls and T2DM

Clinical Characteristic (mg/dL)	Control (n=11) Mean ± SD		Diabetic (n=53) Mean ± SD	
	Pro12Pro	Non Pro12Pro	Pro12Pro	Non Pro12Pro
Fasting plasma glucose (FPG)	102.6 ± 9.4	96.3 ± 13.1	122 ± 38.2	136.4 ± 54.0
Total cholesterol (TC)	196.6 ± 55.1	190 ± 38.7	183.4 ± 48.2	208.7 ± 61.8
High density lipoprotein (HDL)	50.3 ± 15.4	51.2 ± 22.0	36.6 ± 8.9	35.7 ± 11.3
Low density lipoprotein (LDL)	121.4 ± 43.8	120.4 ± 29.5	116.5 ± 44.8	196.1 ± 166.0
Triglycerides (TG)	127 ± 72.1	144 ± 66.6	159.1 ± 102.1	139 ± 97.1

The comparison of the anthropometric and clinical characteristics by the polymorphism PPAR-γ (rs1801282) in controls and diabetic patients revealed no statistically important associations with anthropometric and clinical characteristics as were seen for this polymorphism in controls. Also, no statistically significant ($p > 0.05$) links with anthropometric and clinical characteristics were observed for this polymorphism in controls. It is particularly noteworthy that in diabetic subjects, there were statistically significant differences ($p < 0.05$) in total cholesterol (TC) values, with carriers of the Ala12 allele (non Pro12Pro) having higher TC values than Pro12 homozygotes (See Table 11).

Genotyping using Restriction Fragment Length Polymorphism

Briefly, DNA from the samples was extracted using commercially available kit from Qiagen. PCR amplifications for both genes was performed using specific primers. For Restriction Fragment Length Polymorphism (RFLP) analysis, 1 μ l of PCR product was incubated with 1 μ l of CfoI enzyme (10 U/ μ l, in a final volume of 20 μ l for 1 hour at 37 °C. The products were run on a 2% non-denaturing polyacrylamide gel electrophoresis (PAGE) for 30 minutes at 100 V. Bands were observed after ethidium bromide staining and UV light exposure.

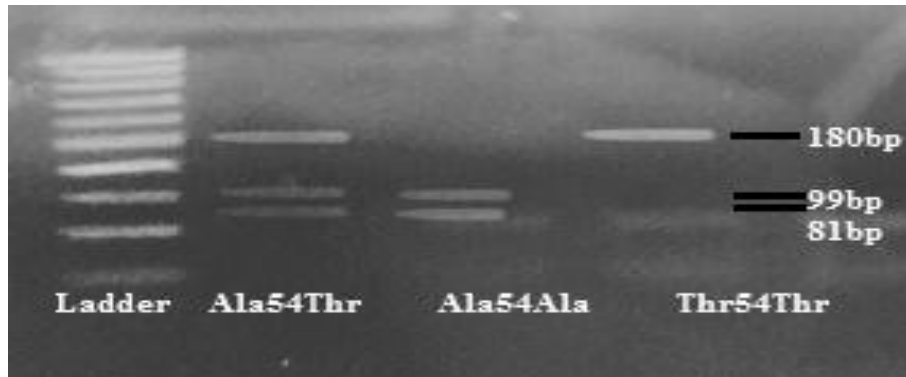


Figure 2: Original representative gel showing polymorphisms in FABP2 gene.

Data from figure 2 shows PCR-RFLP non-denaturing PAGE gel after digestion with CfoI enzyme. The 180 bp band corresponds to the G allele (Ala) and the 99 and 81 bp bands correspond to the A allele (Thr) of the FABP2 gene. The representative gel is typical of 6-7 different experiments.

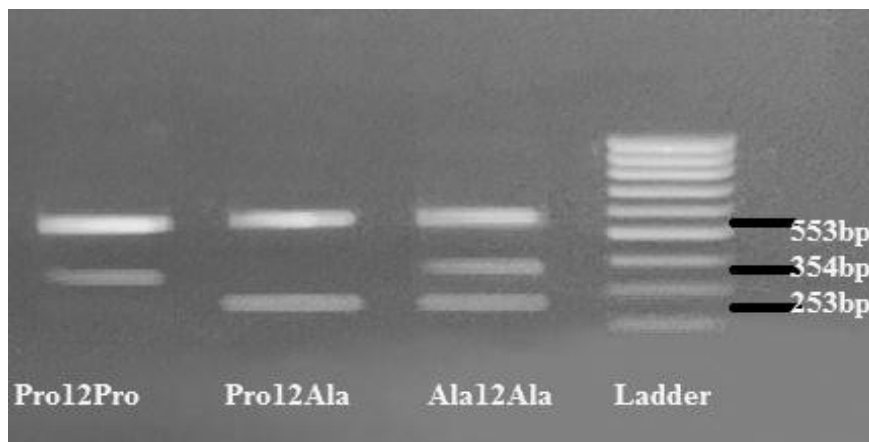


Figure 3: Representative gel showing PPAR- γ genotyping.

Data from figure 3 shows PPAR- γ genotyping by tetra primer ARMS-PCR. The 553 bp band is the product of the outer primers, the 354 bp band, of an outer primer and the inner primer for allele

G (Ala) and the 253 bp band, of the other outer primer and the inner primer for the C allele (Pro). The representative gel is typical of 6-7 different experiments.

Discussion

The role of the FABP2 and PPAR γ polymorphisms in many diseases, including obesity, diabetes, and insulin resistance, has been assessed in different ethnicities, but the results of associations across different populations are contradictory and inconsistent (Tavares et al., 2004; Barbieri et al., 2005; Yilmaz-Aydogan et al., 2011; Alharbi et al., 2014; Raza et al., 2017).

The FABP-2 gene has been contemplated as a possible choice for diabetes because this protein is involved in the absorption and metabolism of fatty acids and may, therefore, affect insulin sensitivity and glucose homeostasis. The most broadly studied variant is the mis-sense Ala54Thr variation, which is common in various populations and results in increased fatty acid absorption in vivo. This is the first study showing the prevalence of Thr54 FABP variant in the United Arab Emirates population.

Comparing the present results with other studies especially in samples of patients from this current study with T2DM, the frequency of the Thr54 allele was 0.27. This value was similar to a Brazilian study that reported a Thr54 frequency 0.25 in T2DM individuals (Almeida et al., 2010). On the other hand, the frequency of the Thr54Thr genotype was 4.00% in T2DM patients in this study, whereas the frequency of the Thr54Thr genotype was 6.25% in Brazilian diabetic patients and 4.00% in American patients with T2DM (Canani et al., 2005).

In order to prove that defects in the FABP-2 gene could be linked with T2DM and their related metabolic traits, the current study investigated the effects of Ala54Thr variant in UAE resident population. Studies investigating the association of FABP-2 A54T polymorphism with T2DM were inconsistent. Several studies have reported the association between the Ala54Thr polymorphism of FABP-2 with insulin resistance and diabetes (Yamada et al., 1997; Chiu et al., 2001; Kim et al., 2001; Duarte et al., 2003; Albala et al., 2012). Other studies have reported no association between this polymorphism and T2DM (Rissanen et al., 1997; Ito et al., 1999; Lei et al., 1999). There are some differences among studies on the design as well as the genetic models used to assess associations of FABP-2 Ala54Thr polymorphism with this disease. The current study investigated the association of FABP-2 Ala54Thr polymorphism with T2DM according to all possible genetic models. No association of FABP-2 Ala54Thr polymorphism was found with T2DM according to any genetic model used in this study, a finding shared by other studies that examined such an association (Weiss et al., 2002).

The present study did not find differences in glycaemia, HbA1c and serum lipids between FABP-2 genotype groups although significant differences in BMI were attained in diabetic group carriers of the Thr allele. The results from studies published on the association of FABP-2 Ala54Thr polymorphism with body mass index (BMI) are conflicting, but the conclusions from the meta analysis carried out by (Zhao et al., 2011) showed no evidence that the FABP-2 Ala54Thr polymorphism is significantly associated with BMI in overall populations. It is possible that carriers of the Thr allele in the T2DM patient confer some degree of susceptibility to obesity, associated with an influence of gene/environment interactions such as diet, exercise, body composition and life style modification (de Luis et al., 2006).

In this study, the frequencies of the Ala allele of the PPAR- γ polymorphism in participants with and without diabetes were 1.3% and 5%, respectively. The frequency of the Ala allele appears to vary greatly by the genetic background of the populations (Huguenin et al., 2010). In general, the frequency of the Ala allele has been reported to be highest in Caucasians (Stumvoll et al., 2002). Variations in the allelic frequency of the Pro12Ala polymorphism across different ethnicities and regions could be attributed to genetic variations and to different environmental and lifestyle exposures (Lindi et al., 2001).

The present study also revealed that the Ala allele of the Pro12Ala polymorphism was associated with a significantly lower risk of T2DM in UAE population. Consistent with the current results, Altshuler et al., (2000) found a significant decrease in diabetes risk associated with the Ala allele in a Caucasian population. In Finnish subjects, the Ala12 variant of the PPAR- γ gene was associated with protection against T2DM. Meta-analyses (Ludovico et al., 2012) also showed a significant effect of the Ala allele on lower development of T2DM.

The results of the current study did not find any major differences in glycaemia, LDL-c, HDL-c and TG between PPAR- γ genotype groups even though considerable differences in TC were present in diabetic group carriers of the Ala allele. Mori et al., (2001) reported that carriers of the Ala 12 allele had a higher cholesterol level compared to those that did not have the allele among diabetic subjects. A study done on an Italian population found that this polymorphism was not related with anthropometrical and biochemical parameters among non-glycaemic and diabetic subjects (Mancini et al., 1999).

The substitution from proline to alanine at codon 12 has been shown to regulate transcriptional activity (Masugi et al., 2000). Because this polymorphism is next to the amino-terminus of the protein in the ligand-independent activation domain, its activity is induced by insulin through

phosphorylation. Alanine helps in the formation of helices, but proline prevents it. Thus, it is possible that a change in this amino acid affects the structure and consequently the function of the protein. The alanine isoform contributes to less efficient stimulation of PPAR- γ target genes and predisposes people to reduce levels of adipose tissue mass accumulation. This in turn may improve insulin sensitivity. It is well known that decreased insulin sensitivity plays an important role in the pathogenesis of T2DM. Although a lot of data on individual gene variants that affect lipid and lipoprotein metabolism is present in literature, they are worthy for identifying individual profiles for T2DM risk and its altered protein is quite limited. This is due to the small effect that a single gene has, that can also vary depending on other factors such as lifestyle, environmental stimuli, and other genes.

It was initially presumed that rs1801282 polymorphisms (Pro12 allele) may influence the receptor activity, the ability to transactivate responsive promoters and so on to regulate the key target genes of PPAR- γ which could influence the lipid metabolism. But the specific biological mechanism needs to be further studied. On the other hand, FABP-2 is involved in the transport and metabolism of saturated and unsaturated long chain fatty acids (FAs). The mis-sense Ala54Thr variation results in increased activity. Thus, subjects with the Thr54 allele may have increased intestinal absorption of cholesterol, and this is associated with the higher cholesterol and LDL cholesterol levels in those with Thr54 allele among diabetic patients.

In conclusion, the results from the present study show a distinct association of the Ala54Thr polymorphism of FABP-2 gene with T2DM in several nationalities residing in the United Arab Emirates. This variant is a probable contributor to the risk of T2DM and problems related to it. The findings of this study appear to be in agreement with that of several other studies. One among such study done on the Guadeloupian population, concluded a statistically important association between the Ala54Thr polymorphism and T2DM as well as the physiological function of the FABP2 gene (Lepretre et al., 1999). Another study done on the Mexican-American population also confirmed the association of the Ala54Thr with diabetes and its related problems. A study on the Pima Indian population also came to the conclusion that the polymorphism in these genes was associated with increased fatty acid binding and increased fat oxidation, thus being linked with insulin resistance (Baier et al., 1995).

Furthermore, this study showed that the Pro12Ala polymorphism had no significant effect on T2DM and did not pose as a risk factor for dyslipidemia. No association between Pro12Ala polymorphism and T2DM was found in the population sample of various nationalities residing in

the United Arab Emirates. The findings of the present study are in agreement with the results of previous studies in which no direct influence of the PPAR- γ Pro12Ala polymorphism genetic variation in the development of T2DM was found (Tavares et al., 2004; Gallicchio et al., 2008; Lv et al., 2017).

In conclusion, several limitations of this study should be considered. Firstly, more SNPs of FABP2 and PPAR-gene should have been chosen to capture maximum genetic information. Secondly, there was a relatively small sample size in this study, other larger sample studies should be conducted in the future research.

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Publications and Presentations

Shalini Behl, Abdu Adem, Arif Hussain and Jaipaul Singh (2019). Effects of rilpivirine, 17 β -estradiol and β -naphthoflavone on the inflammatory status of release of adipocytokines in 3T3-L1 adipocytes in vitro. Molecular Biology Reports <https://doi.org/10.1007/s11033-019-04671-4>.

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Best Poster Award

Role of Rilpivirine and 17 β -Estradiol in potentiating the risk of drug-induced obesity.

Shalini Behl, Aisha Rauf, Arif Hussain, Jaipaul Singh.

Presented at "4th Current Trends in Biotechnology" held at Manipal Academy of Higher Education,Dubai campus on 18-19th of April,2018.

Oral Presentation

In vitro studies on adipose cells treated with rilpivirine and quercetin: Effect on triglyceride accumulation, release of adipocytokines, oxidative stress and protein markers.

Shalini Behl, Abdu Adem, Arif Hussain and Jaipaul Singh.

Presented at "5th Current Trends in Biotechnology" held at Manipal Academy of Higher Education,Dubai campus on 10-11th of April,2019.

